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THE IM-9 CELL LINE: A MODEL FOR EVALUATING TCDD-INDUCED
MODULATION OF THE POLYMORPHIC HUMAN HS1,2 ENHANCER WITHIN
THE 3' IMMUNOGLOBULIN HEAVY CHAIN REGULATORY REGION.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

RUTH CHAMBERS-TURNER
B.S., Central State University, 2003

2010
Wright State University

WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Date 3/18/2010

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Ruth Chambers-Turner ENTITLED The IM-9 cell line: a model for evaluating TCDD-induced modulation of the polymorphic human hsl,2 enhancer within the 3' immunoglobulin heavy chain regulatory region. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Chambers-Turner, Ruth M.S., Microbiology and Immunology Graduate Program, Wright State University, 2010. The IM-9 cell line: a model for evaluating TCDD-induced modulation of the polymorphic human hs1,2 enhancer within the 3' immunoglobulin regulatory region.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a disrupter, of B-cell differentiation, induces binding of the aryl hydrocarbon receptor (AhR) nuclear complex to dioxin responsive elements (DRE) within the mouse immunoglobulin heavy chain regulatory region (3'IgHRR), and produces a marked inhibition of 3'IgHRR activation, IgH expression, and antibody secretion in a well-characterized mouse B-cell line (CH12.LX). The mouse 3'IgHRR consists of at least four enhancers (hs3a; hs1,2; hs3b; hs4), and is highly homologous with the three enhancers (hs3; hs1,2; hs4) of the human 3'IgHRR. A polymorphism of the human hs1,2 enhancer (resulting in varying numbers of tandem repeats containing a DRE and κ B site) has been correlated with several autoimmune diseases. Although the human and mouse hs1,2 enhancers share a ~90% identity, luciferase reporter studies in mouse CH12.LX B-cells showed that TCDD inhibited LPS stimulation of the mouse hs1,2 enhancer but co-treatment with TCDD and LPS synergistically activated human hs1,2 enhancer activity. To evaluate transcriptional differences between the human and mouse hs1,2 enhancers, our objectives were to

characterize the IM-9 cells as a potential human B-cell model, and to evaluate TCDD-induced transcriptional regulation of the polymorphic human hs1,2 enhancer in a human cell line. We confirmed AhR expression and TCDD-induced CYP1A1 induction in IM-9 cells. Then we transiently transfected IM-9 cells with the human hs1,2 reporters and determined that TCDD activates the human hs1,2 enhancer in IM-9 B-cells, as seen in CH12.LX B-cells. However, the TCDD-induced fold-activation in human IM-9 cells appeared less compared to results in mouse CH12.LX B-cells perhaps due to differences between the mouse and human AhR. Our data suggests that the TCDD-induced inhibition of the mouse hs1,2 enhancer versus the activation of the human hs1,2 enhancer may be related to an inhibitory BSAP site located on the mouse hs1,2 enhancer that is absent from the human hs1,2 enhancer. Our results support the use of IM-9 cells as a model for studies evaluating mechanistic differences between the mouse and human hs1,2 enhancers.

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INTRODUCTION

Overview of B-cell and immunoglobulin development

2-3-7-8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental contaminant that has demonstrated toxic effects in laboratory animals and humans. One of the most sensitive targets of TCDD is B cells in which TCDD inhibits expression of the immunoglobulin heavy chain gene (IgH) and secretion of immunoglobulin (Ig) in activated mouse B cells (Sulentic et al., 1998; Sulentic et al; 2000; Dooley and Holsapple, 1988; Karras and Holsapple, 1994). Immunoglobulin, also known as antibody (Ab), is composed of two identical heavy and light chains, each containing a constant and variable region. The composition of the heavy and light chains is generated in a complex sequence of events resulting in genetic rearrangement of the immunoglobulin heavy chain (IgH) and light chain (IgL) genes from their germline configurations. Specifically, a single variable (V), diversity (D) (only for IgH), and joining (J) region will be recombined to form the VDJ variable region of IgH and the VJ variable region of IgL. V(D)J recombination of the light and heavy chains will confer antigen specificity of the antibody. The IgH gene contains several constant (C) regions that encode each of the five major classes of antibody (IgM, IgD, IgG, IgA, IgE). A DNA recombination event called class switch recombination (CSR) will determine which isotype is expressed. Rearrangement of the VDJ regions and early IgM expression in the cytoplasm occurs during the first stages of B cell maturation from precursor stem cells to Pro then Pre-B

cells (Fig. 1). During the next phase, the immature B cell stage, early IgM is expressed on the surface of the cell (Fig. 1). In the following mature B cell phase, both IgM and IgD are expressed on the cell membrane (Fig. 1). Notably, if a B cell fails a step in the maturation process or recognizes a self antigen, it goes through negative selection, and will die by apoptosis. Mature B cells can bind antigen through their membrane bound Ig which is called the B-cell receptor (BCR). The B cell will not differentiate further until it has been activated by antigen (Ag). Activation initiates stimulatory pathways that result in regulation of class switch recombination (CSR), proliferation, and Ig expression and secretion. Activation can be initiated in a T-independent or T-dependent manner. In T-independent activation a T-independent antigen binds to the BCR or a toll-like receptor on the B cell membrane. An example of a T-independent antigen is lipopolysaccharide (LPS), a cell wall component of gram negative bacteria that can, in higher doses, induce polyclonal activation and antibody secretion by binding to TLR4 or a LPS-specific BCR. Activation with a T-dependent antigen requires that first the T-dependent antigen binds to the BCR. The antigen is then engulfed and digested into peptides that are subsequently displayed on the B cell surface by major histocompatibility complex II (MHC II) molecules. Then the MHC II/antigen is cross-linked by a CD4⁺ T-cell receptor along with an additional stimulatory interaction between the CD40 receptor on the B cell and CD40 ligand (CD40L) on the T-cell. Activation of a B cell induces a signal transduction pathway that causes B cell proliferation and differentiation to high antibody producing plasma cells or memory B cells (Fig. 1). After a B cell has been activated, additional BCR diversity for antigen can still be generated by somatic hypermutation, in which single nucleotides of the already rearranged VJ or VDJ regions are randomly exchanged

with different nucleotides. Additionally, after B cell activation, CSR can change the isotype of the expressed immunoglobulin. During CSR double stranded DNA is enzymatically broken in two of the repetitive sequence regions, termed switch regions (S), that are associated with each constant region. Then, the segment in between the two broken S regions, one located upstream of the first constant region and one located upstream of the constant region that will be expressed following CSR, is deleted out of the gene and the VDJ is joined to the switched constant region. Throughout the CSR process the IgH chain maintains its VDJ sequence, and therefore, variable region antigen specificity remains unchanged. However, a new constant region will be expressed which will reflect a change in isotype. As Ig is the key effector molecule of the B-cell-mediated immune response, TCDD-induced modulation of Ig expression and secretion is highly significant. Normal Ig expression and secretion is in part, dependent on the complex organization of the IgH gene.

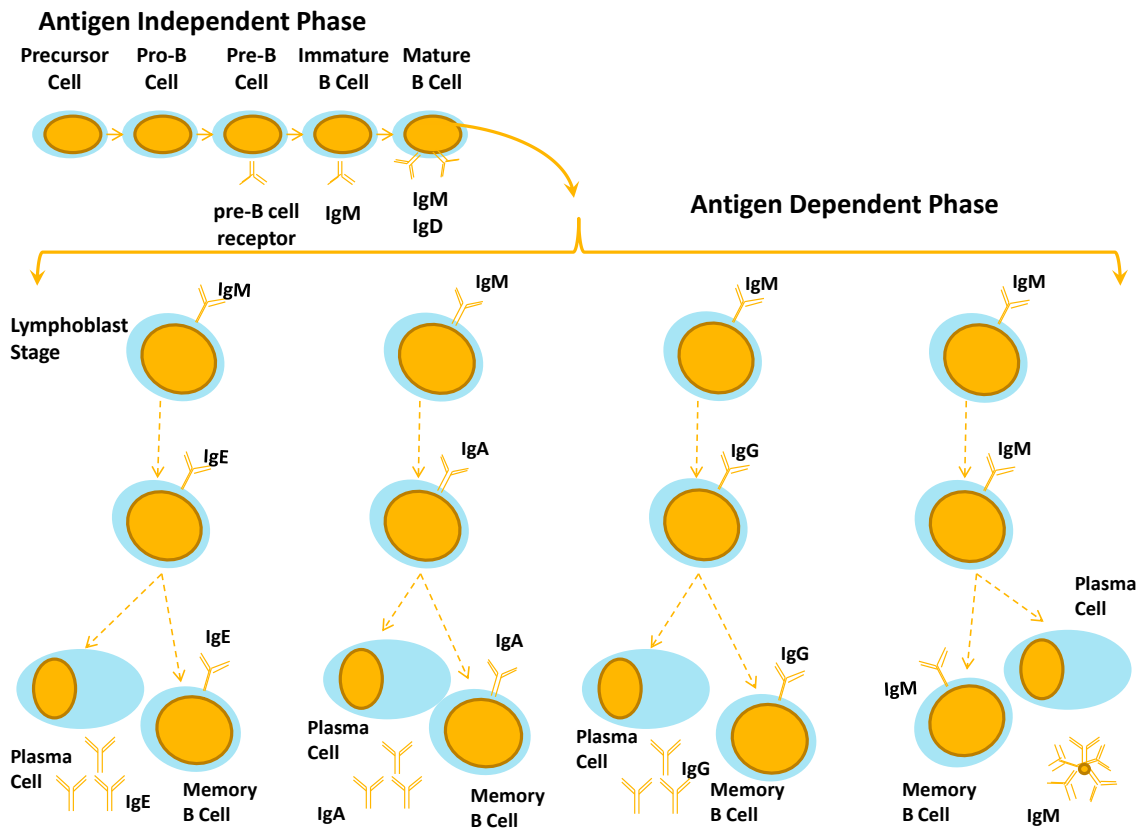


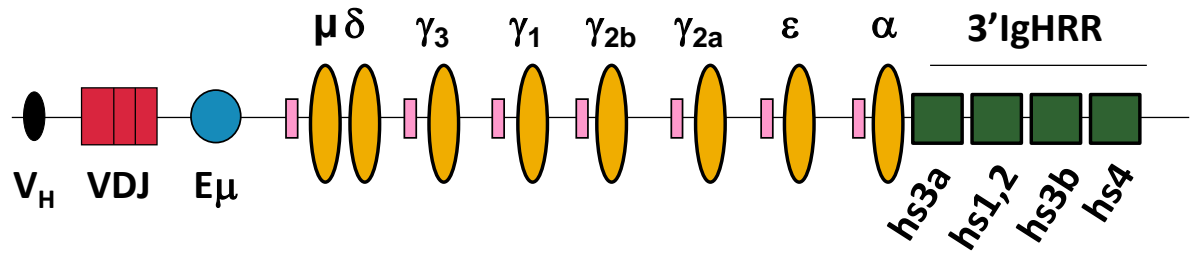
Figure 1. B-cell maturation, differentiation, and Ig expression. B cells mature independent of antigen from precursor stem cells to IgM and IgD-expressing mature B cells. Activation with antigen results in differentiation into B lymphoblast cells, which proliferate polyclonally into high antibody-secreting plasma cells, and membrane bound Ig-expressing memory B cells.

Organization of the immunoglobulin heavy chain gene

Regulation of the complexly organized IgH is carefully orchestrated by several regulatory regions located on both the 5' and 3' ends of the IgH gene. The most 5' element of the IgH is the variable heavy chain promoter (V_H) which is followed by VDJ regions which directly contribute to specificity of the immunoglobulin heavy chain during B cell development (Reviewed by Alt et al. 1992) (Fig. 2). The immunoglobulin heavy chain intronic enhancer (E_μ) is located 5' to the μ constant region and regulates VDJ joining during early B cell development (Serwe and Sablitzky, 1993) (Fig. 2). Initially, the E_μ enhancer was believed to be the lone enhancer of the IgH. However, Serwe and Sablitzky (1993) conducted a study in which embryonic stem cells with a deleted E_μ enhancer were used to produce chimeric mice that generated B cells that, in spite of the deleted E_μ enhancer, still demonstrated V-DJ rearrangements of the IgH loci, although fewer rearrangements were observed than that of control B cells. This study was supported by additional studies in which IgH expression was maintained independent of the E_μ enhancer (Wabl et al, 1984; Klein et al., 1984). Bottaro et al. (1998) supported evidence of a second enhancer region in studies in which hybridomas generated from cells containing a E_μ deletion had significant but incomplete inhibition of CSR in the IgH locus, suggesting partial regulation by a separate regulatory region. Support for a second enhancer region was also established in studies in which CSR was disrupted by the deletion of an enhancer region located about 16 kb 3' of the $C\alpha$ gene in mouse B cells (Patterson et al., 1990; Lieberman et al., 1991; Darizvach et al., 1991). We now know that enhancer as the hypersensitivity site 1,2 (hs1,2) enhancer. In another study, B cells

from $hs1,2^{-/-}$ chimeric mice displayed altered IgG isotype expression and a decrease in corresponding mRNA transcripts after LPS activation as compared to the wildtype mice, suggesting an altered CSR response (Cogné et al., 1994). Recent reports suggest that regulation of the IgH gene is partially mediated via physical interactions between the E μ enhancer and the 3'IgHRR in plasma cells (Ju et al. 2007) and in primary splenic cells VDJ-3'IgHRR as well as E μ -3'IgHRR interactions were reported (Ju et al. 2007). Regulation of the 3'IgHRR is highly complex because it is composed of multiple distinct enhancers (Fig. 2), each containing binding sites for several transcription factors. The mouse 3'IgHRR contains four hypersensitivity sites associated with enhancer activity (hs3a; hs1,2; hs3b; and hs4) (Madisen and Groudine, 1994; Chauveau and Cogné, 1998; Saleque et al., 1997) (Fig. 2). Alternatively, the human IgH contains two 3'IgHRR downstream of two C α regions, C α_1 and C α_2 . The two 3'IgHRR are nearly identical and contain three enhancers, hs3a, hs1,2 and hs4 with no equivalent of the mouse hs3b enhancer (Mills et al., 1997) (Fig. 2). The human C α_1 3'IgHRR demonstrates elevated transcription and expression versus the human C α_2 3'IgHRR (Mills et al., 1997). The difference could be due to a duplication event resulting in the inversion of the C α_2 hs1,2 enhancer with respect to its C α_1 homologue (Mills et al. 1997). Additionally the difference in transcription and expression could be due to the fact that the E μ synergizes with the more upstream C α_1 3'IgHRR, increasing the overall enhancer strength (Mills et al., 1997). The discovery of the 3'IgHRR led to the question of the mechanism of chemically-induced deficiencies in B cell function, one specific chemical mediator being the potent contaminant TCDD.

Mouse IgH locus



Human IgH locus

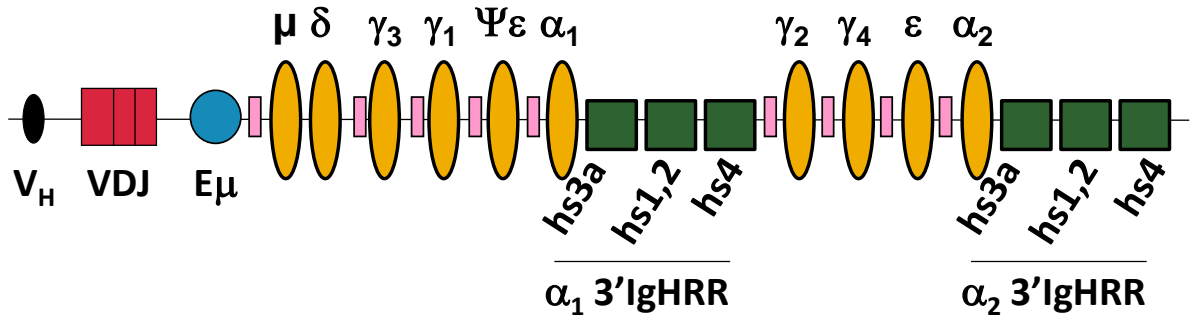


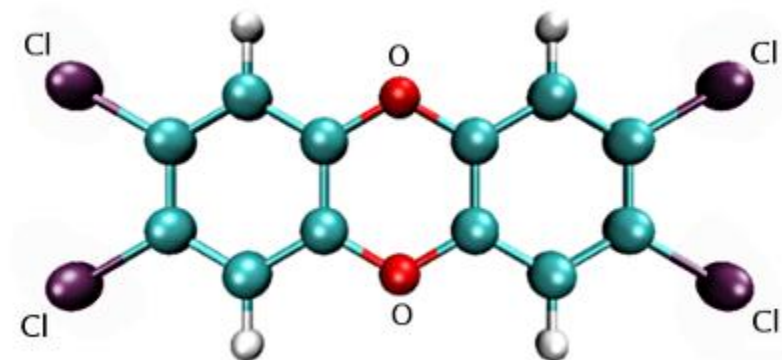
Figure 2. Schematic of the mouse and human IgH genes. Variable heavy chain promoter, V_H , black oval; rearranged VDJ segments, red rectangles; E_μ enhancer, blue circle; S regions, pink rectangles; constant regions, orange ovals, 3'IgHRR green boxes.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an environmentally persistent polycyclic aromatic hydrocarbon (PAH) (Fig. 3) that is produced as a byproduct in the combustion of chlorinated organic compounds (Reviewed by Fiedler, 1996 and Fiedler et al., 2006). One common source of TCDD contamination is its release during the manufacture of commercial products (Reviewed by Fiedler, 1996; Fiedler et al., 2006). Additionally, TCDD is released as a pollutant from natural sources including forest fires and volcanoes (Reviewed by Fiedler, 1996; Fiedler et al., 2006). TCDD is a potential threat to human health due to bioaccumulation in humans and wildlife through multiple sources of exposure (i.e. air, drinking water, soil, dust and smoke) (Reviewed by Fiedler, 1996; Fiedler et al., 2006). Research on the health effects of TCDD was spurred in the 1970's due its use in the infamous Agent Orange. Agent Orange was an herbicide that was sprayed to defoliate the dense terrain of trees, shrubbery, and crops of Vietnam in order to deny enemy cover and food during the Vietnam War (Kashida, et al., 2010). Agent Orange has been associated with prostate cancer, eczema, radiculopathy, diabetes mellitus, peripheral neuropathy, hypertension, and birth defects in exposed individuals (Giri et al., 2004; Kim et al., 2003a, 2003b; Ngo et al., 2006). Agent Orange consists of a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) with TCDD as a contaminant of the 2-4-5-T (Reviewed by Young et al., 2004). Aside from Agent Orange, TCDD has gained much media exposure ever since the 1970's due to environmental disasters in which high amounts of TCDD were accidentally released (Fiedler et al., 2006). More recently TCDD made headlines in 2004 when it was used to poison Viktor Yushchenko, President of the Ukraine, which resulted in chloracne,

a disfiguring skin condition. The pathogenesis of chloracne includes a multitude of acne-like eruptions of comedones, cysts and pustules, and by squamous metaplasia of epithelial cells within the duct of the sebaceous gland which may in part be due to constitutive activation of the TCDD/Aryl hydrocarbon receptor (AhR) pathway (described below) in epithelial cells (Geusau et al., 2001; Tang et al., 2008) (Fig. 3). In addition, TCDD-associated hepatotoxic, carcinogenic, and immunotoxic effects have been reported in humans (Geusau et al., 2001). Furthermore, in laboratory animals, TCDD has produced hepatotoxic, immunotoxic, teratogenic, carcinogenic, and neurotoxic effects (Mandal 2005), in addition to a wasting syndrome and thymic atrophy (Poland and Knutson 1982). We have explored the immunotoxic effects of TCDD and found that when the mouse B cell line, CH12.LX, is activated by the polyclonal activator LPS, TCDD-co-treatment markedly inhibits IgM secretion (Sulentic et al., 1998, 2000). Moreover a decrease in IgM expression was also demonstrated in primary B cells that had been activated with LPS (Dooley and Holsapple, 1988) or anti-IgM (Karras and Holsapple, 1994) and treated with TCDD. Since Ig expression and secretion is regulated by the 3'IgHRR, we believe there could be a correlation between TCDD-induced immunotoxic effects and chemical modulation of the 3'IgHRR. The overall toxic response from TCDD, the most potent of the PAH family of chemicals, has been suggested to be due, in part, to its affinity for the aryl hydrocarbon receptor (AhR) and activation of the AhR pathway (Pohjanvirta et al., 1999).

A.



B.



Figure 3. Polyaromatic TCDD induces chloracne poisoning. A, polyaromatic hydrocarbon chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (http://commons.wikimedia.org/wiki/File:Dioxine_pcdd.png). B, Viktor Yushchenko before (L) and after (R) 2004 TCDD poisoning that resulted in disfiguring chloracne lesions (http://blog.kievukraine.info/2007_07_01_archive.html).

TCDD, the aryl hydrocarbon receptor, and dioxin response elements

The principle mechanism of TCDD toxicity is thought to be mediated through the AhR pathway. AhR studies were initially derived from research on the inducibility of the metabolic enzyme aryl hydrocarbon hydroxylase (AHH), which is also known as CYP1A1, of the cytochrome P450 superfamily of enzymes. Early AHH inducibility studies utilized mice that were able (responsive), or unable (non-responsive) to induce AHH and therefore metabolize PAH, benzo[a] pyrene (BP) or 3-methylcholanthrene (3-MC) (Reviewed by Whitlock, 1990). However, when TCDD was established as a much more potent inducer of AHH than BP and 3-MC, ligand binding studies using [³H]-TCDD in the responsive mouse strain were conducted to determine the receptor responsible for the induction. In these ligand binding studies an intracellular hepatic protein that could saturably bind TCDD with high affinity was discovered, and named the aryl hydrocarbon receptor (AhR) (Reviewed by Whitlock, 1990). The AhR has now been extensively characterized as a basic helix loop helix protein (bHLH) in the period/aryl hydrocarbon nuclear translocator protein/single minded (Per/ARNT/SIM) family. The Per/ARNT/SIM family of proteins is reportedly involved in regulation of genes controlling circadian rhythm, neurogenesis, stress response to hypoxia, and metabolism (Hogenesh et al., 1997; Hoffman et al., 1991; Crews et al., 1999; Liu et al., 2003). AhR has specifically been identified as a mediator of several cellular processes including regulation of cell cycle, inflammation, and metabolism of xenobiotics including, TCDD (Whitlock et al., 1999; Vogel et al., 2007; Singh et al., 2007; Ito et al., 2004; Bock and Khole et al., 2006). In addition to PAH environmental pollutants, the AhR is responsive

to halogenated hydrocarbons, and planar polychlorinated biphenyls, and recently, several naturally occurring dietary and endogenous ligands have been identified (Reviewed by Denison and Nagy, 2003). The AhR pathway has been extensively characterized using TCDD, the prototypic AhR ligand (Reviewed by Ma, 2001). Similar to the steroid signaling pathway, the AhR pathway begins when lipophilic TCDD crosses the cellular membrane. It binds to AhR, which is in a complex with two heat shock proteins (hsp90) and X-associated protein 2 (XAP2), and the co-chaperone protein p23 (Ma and Whitlock, 1997; Carver et al., 1997; Perdew et al., 1991; Petrulis et al., 2003; Shetty et al., 2003). The AhR complex of proteins and TCDD cross the nuclear membrane where AhR and TCDD dissociate from the protein complex and forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) which is also a bHLH protein (Hord and Perdew, 1994; Hoffman et al., 1991; Probst et al., 1993; Hankinson et al., 1995). The AhR/ARNT heterodimer is believed to induce transcriptional modulation by binding to dioxin response element (DRE) binding sites. Interestingly, AhR has been reported to bind DRE sites in the presence and absence of exogenous ligand (Ma et al., 1997). DRE binding sites consist of the core pentanucleotide sequence, GCGTG, and are well conserved throughout several mammalian genomes (Sun et al., 2004; Tijet et al., 2006). Several DRE sites have been identified in the enhancer region of the metabolic gene CYP1A1, the most studied target of TCDD (Whitlock et al., 1990; Masten and Shiverick, 1995). Indeed, induction of CYP1A1 is the well established gold star indicator of a functional AhR pathway. In a study examining the role of the AhR in the effects of TCDD on IgM, the induction of CYP1A1 was compared to inhibition of IgM expression in activated CH12.LX cells. The findings indicated a structure activity relationship in the

induction of CYP1A1 and the inhibition of IgM expression and secretion in that higher affinity AhR ligands produced a greater effect on these endpoints (Sulentic et al., 2000). Furthermore, statistical analysis of concentration-response curves comparing IC₅₀ values for μ expression and IgM secretion to EC₅₀ values of CYP1A1 induction were not significantly different further suggesting a common mechanism of action, i.e. the AhR pathway (Sulentic et al., 2000). Moreover, we identified DRE-like motifs in the hs1,2 and hs4 enhancers of the mouse 3'IgHRR (Sulentic et al., 2000) and EMSA-Western studies have confirmed binding of AhR nuclear protein from TCDD-treated CH12.LX cells to oligomers designed from the mouse hs1,2 and hs4 enhancer DRE sites (Sulentic et al., 2000). Likewise, ChIP analysis confirmed AhR-DRE binding complexes in the hs4 domain of the mouse 3'IgHRR (Sulentic et al., 2004a). Thus, we believe that TCDD may induce AhR/ARNT binding to DRE sites of the 3'IgHRR which, in turn, modulates transcriptional regulation of Ig expression and secretion.

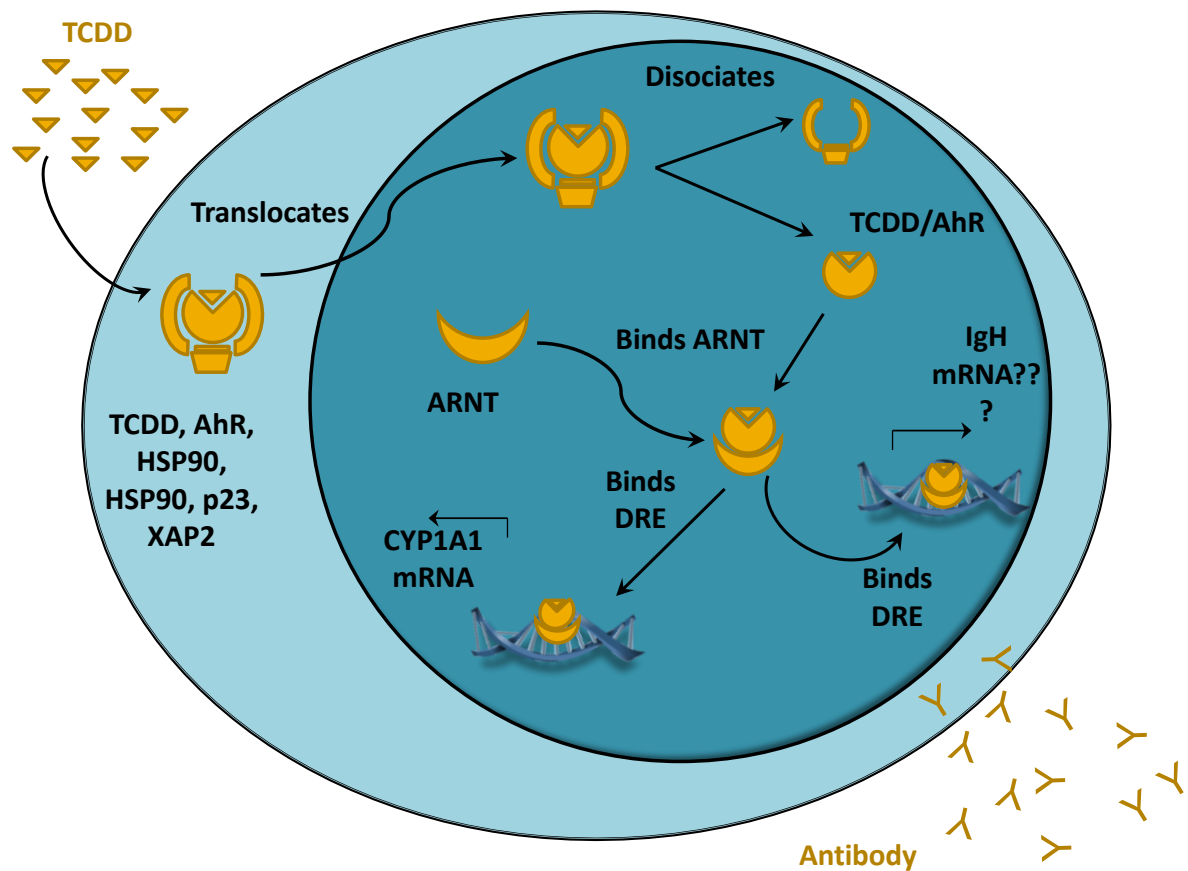


Figure 4. The Aryl hydrocarbon pathway. TCDD enters the cell then binds with the AhR complex consisting of XAP2, p23, two HSP90 proteins. The complex translocates to the nucleus where the XAP2, p23, and HSP90 proteins dissociate from TCDD/AhR. The TCDD/AhR then forms a heterodimer with ARNT then binds to DRE sequences of the CYP1A1 gene and possibly the IgH gene which may modulate Ig expression and secretion.

Transcriptional modulation of the 3'IgHRR by TCDD

In order to examine the effects of TCDD on transcriptional regulation of the 3'IgHRR, we previously utilized luciferase reporter constructs containing the mouse variable heavy chain promoter and the mouse 3'IgHRR, hs4 or hs1,2 enhancers (Sulentic et al., 2004a, 2004b; Fernando et al., manuscript in preparation). In these experiments we found that while TCDD and LPS co-treatment synergistically activate the hs4 enhancer, the activities of the mouse hs1,2 enhancer and the entire mouse 3'IgHRR were inhibited by TCDD in LPS-activated mouse CH12.LX cells (Sulentic 2004a, 2004b). In a separate study, a cell line was generated in which CRE-loxP technology was utilized to delete the hs3b and hs4 enhancers from a mini locus containing a 3'IgHRR-regulated γ 2b transgene, therefore, leaving the γ 2b transgene under the regulation of the hs3a and hs1,2 enhancers (Fernando et al., manuscript in preparation). This study demonstrated that while LPS activated γ 2b expression in the control cell line containing the complete 3'IgHRR-regulated mini locus as well as the hs3a/hs1,2-regulated mini locus, the two mini loci were equally inhibited by TCDD which suggests that the TCDD-induced inhibition of the mouse 3'IgHRR in LPS-activated mouse B cells is mediated through the hs1,2 enhancer, (and possibly the hs3a enhancer) (Fernando et al., manuscript in preparation).

Interestingly, whereas LPS-induced activation of the mouse hs1,2 enhancer is inhibited by TCDD, the human hs1,2 enhancer demonstrates synergistic activation in LPS-activated and TCDD co-treated mouse CH12.LX cells (Fernando et al., manuscript in preparation). These studies clearly demonstrate the apparent difference between the effects of TCDD on the mouse versus human hs1,2 enhancers. Moreover, our previous studies suggest that each enhancer of the 3'IgHRR is individually regulated and support

the theory that multifaceted transcriptional regulatory events are contributing to the effects of TCDD on Ig expression and secretion in B cells.

Basal transcriptional regulation of the individual enhancers of the mouse and human 3'IgHRR

Over the last 20 years the basal transcriptional regulatory events of the 3'IgHRR have been outlined in human and mouse B cells. Indeed, several breakthrough studies have used the DNA fragmentation method in which restriction enzymes are utilized to map the individual enhancers of the mouse and human 3'IgHRR (Madisen and Groudine, 1994; Chauveau et al., 1998; Mills et al., 1997; Kanda et al., 2000). In these studies transient and/or stable transfections were conducted using the enhancers of the 3'IgHRR cloned into reporter constructs of a promoter and the human growth hormone gene (Madisen and Groudine 1994), chloramphenicol acetyl transferase gene (Chauveau et al., 1998), or a luciferase gene (Mills et al., 1997, Kanda et al., 2000). In each study an enhancerless reporter containing only the promoter was used as a negative control (Madisen and Groudine, 1994; Chauveau et al., 1998; Mills et al., 1997; Kanda et al., 2000). The early studies established that the human and mouse hs1,2 enhancers were most transcriptionally active in late B cell lines such as mature B cells and plasmacytomas (Madisen and Groudine, 1994; Chauveau et al., 1998; Mills et al., 1997; Kanda et al., 2000). Moreover, the hs4 is the only enhancer that exhibited enhancer activity in pre and pro-B cells as well as in the later phases of B cell development (Madisen and Groudine, 1994; Chauveau et al., 1998; Giannini et al., 1993; Michaelson et al., 1996). Two studies utilizing human and mouse hs3 enhancers (hs3a and hs3b in

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mouse studies) found that, alone, the hs3 enhancer had little or no activity (Chauveau et al., 1998; Mills et al., 1997). However, the hs3a and hs3b enhancers flanking the mouse hs1,2 enhancer were determined to be palindromes (Salaque et al., 1997) and a construct of the three mouse enhancers in tandem (hs3a←hs1,2→hs3b) generated the greatest activity in plasma cells compared to the individual enhancers (Chauveau et al., 1998). Furthermore, other studies agreed that, with respect to the individual enhancers, there was substantial activity when all of the enhancers were linked together in tandem in pre-B cells and mature B cells (Madisen and Groudine, 1994; Chauveau et al., 1998; Kanda et al., 2000). Moreover, a remarkable synergistic amplification was discovered when the E μ enhancer was added to constructs with all of the enhancers in tandem (Chauveau et al., 1998).

Several conserved transcription factor binding sites contribute to the basal activity of each individual enhancer region of the mouse and human 3'IgHRR. The mouse hs3a and hs3b enhancers are 97% identical and the mouse hs3a enhancer shares a 74% homology to the nearly identical human hs3 enhancers of the human C α 1 and C α 2 3'IgHRR over a 200bp core segment (Mills et al., 1997; Chauveau and Cogné, 1996). The hs3 enhancers are the least studied of the 3'IgHRR due to their weak enhancer activity (Mills et al., 1997; Chauveau et al., 1998; Madisen and Groudine 1994), however, sequence analysis has indicated that the mouse hs3a and hs3b enhancers have AP-1 and Oct binding site (Mills et al., 1997, Madisen and Groudine 1994) and the human hs3 enhancers have conserved AP-1 and a SP1 motifs (Guglielmi et al., 2004; Mills et al., 1997). Interestingly, AP-1 binding proteins are activated by TCDD in mouse

hepa1c1c7 cells (Hoffer et al., 1996; Puga et al., 2000) and SP-1 binding has been shown to associate with NF κ B proteins (Hirano et al., 1998). Similar to the hs3 mouse and human enhancers, the mouse and human hs4 enhancers share a 76% homology (Mills et al., 1997). The mouse and human hs4 enhancers have been reported as strong enhancers in early B-cell lineages, but also in some plasmacytoma cell lines (Kanda et al., 2000; Madisen and Groudine, 1994; Chauveau et al., 1998). Partially contributing to this enhancer activity is an exactly conserved Oct motif. Notably, the mouse hs4 enhancer has a weaker second Oct binding site (Guglielmi et al., 2004, Michaelson et al., 1996). Both of the Oct sites from the mouse hs4 have been confirmed by binding studies in pre-B and B cell lines (Michaelson et al., 1996). Additional contributors to mouse and human hs4 activity include DRE and κ B motifs (Sulentic et al., 2004a, 2004b; Mills et al., 1994; Guglielmi et al., 2004). The κ B sites in the mouse and human hs4 enhancers have been established as necessities for hs4 enhancer activity (Kanda et al., 2000), and one of the κ B sites on the mouse hs4 enhancer overlaps with a DRE site (Sulentic et al., 2000). EMSA-Western binding studies have demonstrated binding of AhR/ARNT and NF κ B to this overlapping DRE and κ B site (Sulentic et al., 2000). Additionally, ChIP assay has identified TCDD-inducible AhR binding, presumably to the DRE, in the hs4 enhancer in CH12.LX cells activated with LPS (Sulentic et al. 2004a). Further, site-directed mutation of the DRE and/or κ B sites on an hs4 luciferase reporter construct demonstrated that the DRE and κ B cooperatively regulate the hs4 enhancer (Sulentic et al., 2004a, 2004b). Interestingly, Cos-7 cells demonstrated AhR-dependant modulation of NF κ B binding activity in TCDD-treated cells and in the absence of AhR ligand (Tian et al., 1999). An additional characteristic of the mouse hs4 enhancer is the presence of a high affinity B

cell activator protein (BSAP) site (Mills et al., 1994), which was confirmed by EMSA binding studies with cold competitor (Michaelson et al. 1996). BSAP, also known as Pax5, negatively regulates early B-cell differentiation in part by repressing lineage-inappropriate genes (Reviewed by Nutt et al., 2001, 2008). As B cells mature BSAP negative regulation maintains B cell identity (Reviewed by Nutt et al., 2008). However, expression of B lymphocyte-induced maturation protein 1 (BLIMP-1) in mature B cells inhibits BSAP repression in an autoregulatory negative feedback loop which results in terminal differentiation of B cells into plasma cells that do not express BSAP (Lin et al., 2002; Mora-López et al., 2007; Delogu et al., 2006). Both the mouse and human hs4 and hs1,2 enhancers are complexly regulated via transcription factor binding to BSAP, κ B, Oct, and possibly DRE sites in the various maturation stages of B cell development. In a pre-B cell line the κ B and Oct sites are activators while the BSAP site is a repressor of the hs4 enhancer (Michaelson et al., 1996) (table 1). Further, the BSAP, κ B, and Oct sites are concerted positive regulators of the mouse hs4 enhancer in a mature B cell line, and in plasma cells κ B is a positive regulator of the hs4 and Oct has no effect on activity (Michaelson et al., 1996) (table 1). Conversely, in the mouse hs1,2 enhancer, BSAP, κ B, and Oct sites exert a concerted repression in mature B cells (Michaelson et al., 1996, Singh and Birshtein 1996) (table 1). However, when the BSAP sites of the mouse hs1,2 enhancers are mutated κ B and Oct are no longer repressive in mature B cells (Michaelson et al., 1996; Singh and Birshtein, 1996) and in the absence of BSAP proteins, as is seen in plasma cells, NF κ B and Oct proteins activate the hs1,2 enhancer (Michaelson et al., 1996; Singh and Birshtein, 1996) (table 1). BSAP basal repression of the hs1,2 enhancer in the early B-cell lineages is consistent with findings that BSAP is an inhibitor of early

B-cell differentiation (Nutt et al., 1999). Additionally, the reduced BSAP expression in mature B cells to no expression in plasma cells (Reviewed by Nutt et al., 2001) could relate to the change from repression to activation by NF κ B and Oct proteins. Notably, when mouse splenocytes and the mature B-cell line, CH12.LX, were co-treated with LPS and TCDD, they demonstrated prolonged expression of BSAP that was correlated with a decrease in IgH transcripts (Yoo et al., 2004; Schneider et al., 2008) which suggested that the maintained negative regulation from BSAP could have inhibited the change in role of Oct and NF κ B proteins from repressive to activating factors. Furthermore, as stated above the κ B and DRE sites of the hs4 enhancer have been found to cooperatively influence transcriptional regulation in TCDD-treated and LPS-activated B cells (Sulentic et 2004a, 2004b). Moreover, there is a possible κ B site adjacent to a DRE sites of the hs1,2 enhancer which may also collectively influence transcriptional regulation of the hs1,2 enhancer. Thus, the DRE, κ B, and Oct, in addition to the BSAP sites of the mouse hs1,2 enhancer could be contributing to the inhibitory effect of TCDD and LPS co-treatment in mature B cells. Interestingly, the human hs1,2 enhancer, which shares a 90% homology over a 135 bp core sequence with the mouse enhancer, lacks a BSAP binding site (Mills et al., 1997), and is activated by LPS and TCDD co-treatment rather than inhibited as seen with the mouse hs1,2 enhancer.

Mouse hs4 enhancer	BSAP	κB	Oct
Pre-B cell	↓	↑	↑
Mature B cell	↑	↑	↑
Plasma Cell	*	↑	—

Mouse hs1,2 enhancer	BSAP	κB	Oct
Mature B cell	↓	↓	↓
Plasma Cell	*	↑	↑

Table 1. Regulation of the mouse hs4 and hs1,2 enhancers. Activation (↑), inhibition (↓) or no change (-) in basal activity of the hs4 and hs1,2 enhancers of the 3'IgHRR by transcription factor binding to BSAP, κB, or Oct sites, as determined by site directed mutation or deletion results of studies in pre-B, mature B, or plasma cell lines. The hs1,2 enhancer has low basal activity in pre-B cells which is the likely reason that it was not evaluated. BSAP has been reported to not be expressed in plasma cell lines, which is denoted by “*.”

Transcriptional regulation of the human hs1,2 enhancer

Although, the mouse and human hs1,2 enhancers are highly homologous, the human hs1,2 enhancer has key sequence differences (figs. 5, 6) which may contribute to the TCDD-induced activation versus inhibition seen in the human versus mouse enhancers. Unlike the mouse hs1,2 enhancer, four alleles of the human hs1,2 enhancer have been identified, the $\alpha 1A$, $\alpha 1B$, $\alpha 1C$, and $\alpha 2$, each containing varying numbers of 53bp tandem repeat (Denizot et al., 2001; Chen and Birshstein, 1997; Mills et al., 1997). The $\alpha 1A$ (the wild type of the human allelic hs1,2 enhancer), $\alpha 1B$, and $\alpha 1C$ alleles are polymorphisms of the C $\alpha 1$ hs1,2 enhancer and contain one, two, and three repeats, respectively. The $\alpha 2$ allele is derived from the C $\alpha 2$ hs1,2 enhancer and contains four 53bp repeats (Mills et al. 1997; Denizot et al. 2001). Each 53bp repeat contains a DRE, NF κ B, Ap-1, and two Sp-1 binding sites (Fig. 6) excluding the $\alpha 1C$ allele which does not contain a third repeat of the complete NF κ B and DRE binding sites (Denizot et al., 2001; Chen and Birshstein, 1997; Mills et al., 1997; Fernando et al., manuscript in preparation). Additionally, each repeat is flanked by an Oct site and an AP-1 site overlapping an Ets site (Denizot et al., 2001; Chen and Birshstein, 1997; Mills et al., 1997) (Fig. 6). Luciferase reporters consisting of the variable heavy chain (V_H) promoter alone, or the V_H promoter linked to the $\alpha 1A$, $\alpha 1B$, or $\alpha 1C$ reporter, the human reporters demonstrated an increase in total activation by LPS in increasing order of 53bp repeats, however, excluding the $\alpha 1C$ there was no difference in fold change relative to the basal activity (Fernando et al., manuscript in preparation). When the human hs1,2 enhancers were transfected into mouse CH12.LX cells and co-treated with LPS and TCDD, a synergistic

activation was observed, which also demonstrated an increase in activation according to number of repeats (Fernando et al., manuscript in preparation). Thus this activation appears to be related to the 53bp repeats and their associated transcription factor binding sites. Notably, the LPS and TCDD-induced fold-change of the $\alpha 1C$ reporter is not increased to the extent of the $\alpha 1A$, possibly due to the lack of the DRE and NF κ B binding sites in the third 53bp repeat, and the $\alpha 1B$ had the greatest fold activation (Fernando et al., manuscript in preparation). Furthermore, the $\alpha 1B$ reporter has been associated with several human autoimmune diseases including: IgA nephropathy (IgAN) (Aupetit et al., 2000), Celiac disease, systemic sclerosis, schizophrenia dermatitis herpetiformis, plaque psoriasis (Frezza et al., 2004, 2007, 2009), psoriatic arthritis (Cianci et al., 2008), and rheumatoid arthritis (Tolusso et al., 2009). These studies suggest a correlation with the $\alpha 1B$ allele of the hs1,2 enhancer and immune deregulation of the IgA1 isotype (Aupetit et al., 2000), which could be related to altered regulation of the transcription factors in the 53bp repeat. Another recent study was conducted in which the DRE, NF κ B, AP-1, and/or Oct binding sites were deleted from the $\alpha 1A$ allele of the human hs1,2 enhancer in multiple combinations of deletions (DRE; κ B; DRE and κ B; Oct; DRE, κ B, AP-1; DRE, κ B, AP-1 and Oct) (Fernando et al., manuscript in preparation). This study demonstrated that in TCDD and LPS co-treated mouse CH12.LX cells, the NF κ B binding site was a positive regulator of the TCDD-induced activation and the DRE was a slightly negative regulator (Fernando et al., manuscript in preparation). Additionally, the activation effect of TCDD was not completely abrogated until the deletion of the entire 53bp region (DRE, NF κ B, AP-1) and

the Oct site in the flanking region (Fernando et al., manuscript in process). These results suggest that these sites of the 53bp and flanking regions may collectively contribute to the overall positive regulation of TCDD-induced activation of the human hs1,2 enhancer. However, these studies of the human hs1,2 enhancer were conducted in the mouse CH12.LX cell line. Since AhR endogenous expression and affinity to TCDD is species specific (Reviewed by Connor and Aylward, 2006), the true effect of TCDD on the human hs1,2 enhancer in a human B cell line is unknown.

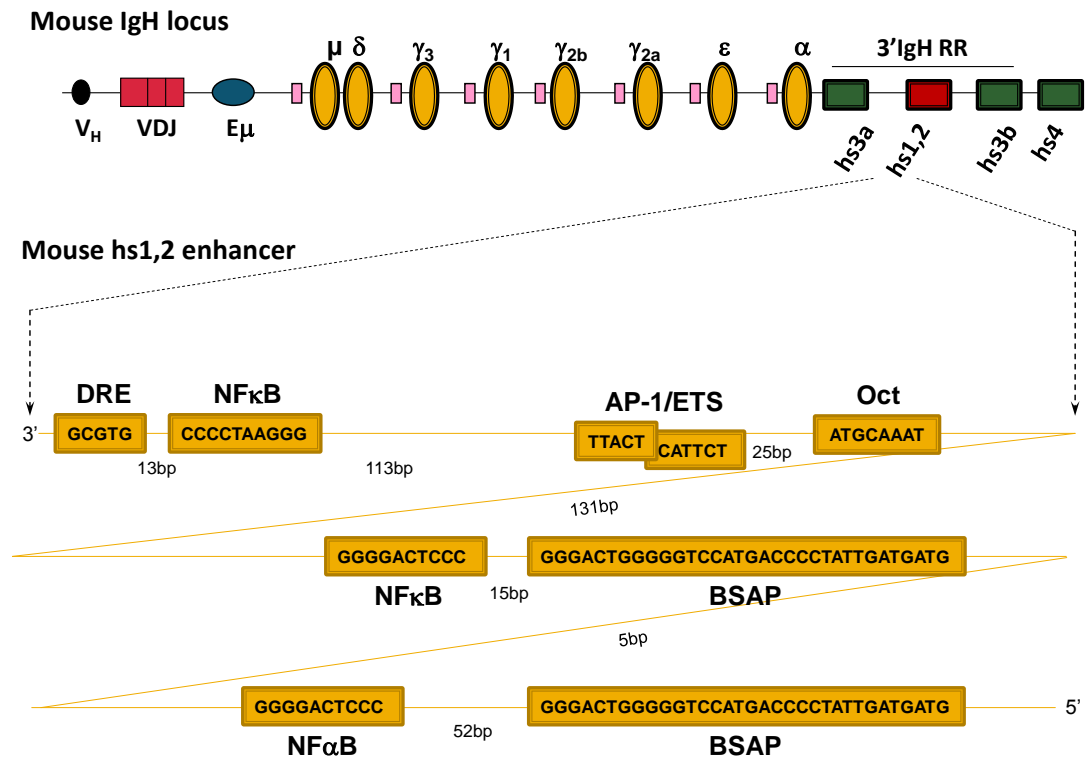


Figure 5. Schematic of the mouse IgH locus and the mouse hs1,2 enhancer. The mouse IgH locus consisting of the V_H promoter, black oval; rearranged VDJ segments, red squares; E_μ enhancer, blue circle; S regions, pink rectangles; constant regions, orange ovals, hs3a, hs3b, and hs4 enhancers, green rectangles and hs1,2 enhancer, red rectangle. Dotted lines point to an enlarged map of the hs1,2 enhancer depicting the relative location and sequence of the transcription factor binding sites of the mouse hs1,2 enhancer.

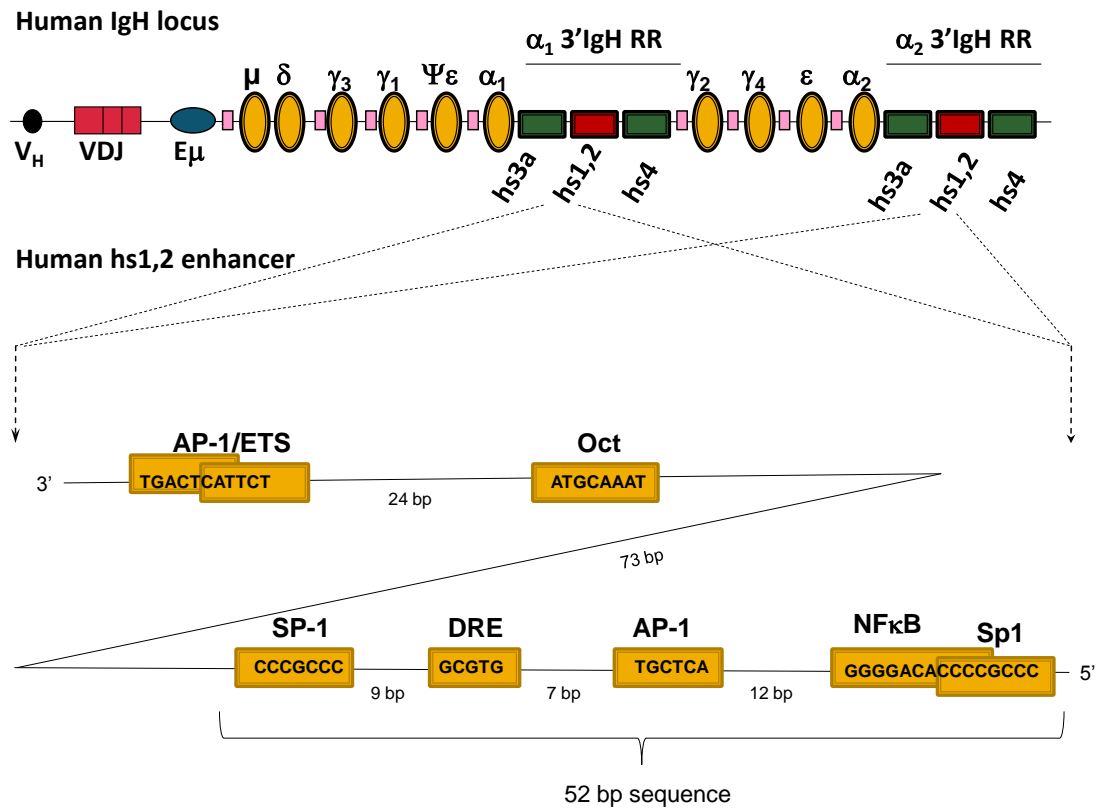


Figure 6. Schematic of the human IgH locus and the α_1 and α_2 hs1,2 enhancer regions. The human IgH consisting of the V_H, black oval; rearranged VDJ segments, red squares; Eμ enhancer, blue circle; S regions, pink squares; constant regions, orange ovals, hs3a, and hs4 enhancers, green rectangles and hs1,2 enhancer, red rectangle. Dotted lines point to an expanded depiction of the nearly identical α_1 and α_2 hs1,2 enhancers and the relative location and sequence of the transcription factor binding sites of the human hs1,2 enhancer. The bracket indicates the transcription factor binding sites included in the 53 bp repeated polymorphism of the human hs1,2 enhancer.

The human IM-9 cellular model

To date several studies have led us to enquire the effects of TCDD on the human hs1,2 enhancer in a human cell line. TCDD-induced effects mediated by the AhR have been extensively characterized in several cellular models including primary B cells (Crawford et al., 1997; Marcus et al., 1998). Also, numerous mouse and human cell studies have examined the 3'IgHRR. Further, the mouse CH12.LX B cell line has been utilized to study the inhibitory effect of TCDD on antibody secretion and Ig expression in LPS-activated cells (Sulentic et al., 1998, 2000). In addition, the TCDD-induced modulation of the mouse hs4 and hs1,2 enhancers and the 3'IgHRR in total has been examined in LPS-activated mouse CH12.LX cells (Sulentic et al., 2000, 2004a, 2004b). Moreover, we have recently completed several studies on the effects of TCDD on transcriptional regulation of the human hs1,2 enhancer in LPS-activated mouse CH12.LX cells (Fernando et al., manuscript in preparation). However, although ligand-activated AhR has been characterized in human primary B cells (Allan and Sherr 2005), a gap remains in our understanding of the effects of TCDD on transcriptional regulation of the human hs1,2 enhancer, possibly through the AhR pathway, in a human B cell line. The human hs1,2 enhancer is of particular interest due to the association of the $\alpha 1B$ allele with several human autoimmune diseases. In order to translate our mouse findings into relevant human application it is necessary to study the effects of TCDD on the human hs1,2 enhancer within a human B cell model. The IM-9 cell line is a potential model for such studies. The IM-9 cell line is reported by American Type Culture Collection to be a CD19⁺ human IgG secreting lymphoblastoid cell line. IgG secretion of the IM-9 cell line

has been characterized by Fahey, Buell, and Sox (1971). Furthermore, Masten and

Shiverick (1996) demonstrated binding of nuclear protein from IM-9 cells to DRE oligomers which were supershifted with anti-AhR antibodies, suggesting functional AhR protein. Notably, the same group found that the BSAP sites of the CD19 gene and the 5' S region of the γ 2a constant region on the IgH chain each have BSAP binding motifs which contain an embedded consensus region of the core DRE sequence (Masten and Shiverick, 1995). Utilizing EMSA analysis they demonstrated that binding of IM-9 nuclear protein to a labeled oligomer consisting of a CYP1A1 DRE is partially inhibited by an unlabeled oligomer corresponding to the BSAP binding site of the CD19 gene, suggesting that AhR binds to the DRE embedded in the BSAP binding sites (Masten and Shiverick, 1995). Thus, the objective of this study was to 1) characterize the expression and function of the AhR in IM-9 cell line for use in mechanistic studies evaluating TCDD-induced effects on the human hs1,2 enhancer, 2) examine the effect of TCDD on the transcriptional regulation of the human hs1,2 enhancer in the human IM-9 cell line, and 3) determine if BSAP is mediating the divergence in transcriptional regulation between the mouse and human hs1,2 enhancers with TCDD treatment.

Relevance

Alteration in the regulation of the 3'IgHRR could pose a significant threat to human health since the AhR is activated by several environmentally persistent ligands, including TCDD, as well as a wide array of chemicals from environmental, dietary, and pharmaceutical origin. In fact, we have recently examined the inhibitory effects of several non-dioxin AhR ligands including, the dietary metabolite indolo(3,2,b)carbazole; the antimalarial drug primaquine; the pesticide carbaryl; and the proton pump inhibitor

omeprazole (Prilosec) on the expression of a stably transfected $\gamma 2b$ transgene under the regulation of the mouse 3'IgHRR in LPS-activated mouse CH12.LX cells (Hensler et al., 2009). Since modulation of the transcriptional activity of the hs1,2 enhancer has been associated with numerous autoimmune diseases, the elucidation of TCDD-induced effects, possibly mediated by the AhR, on the 3'IgHRR is highly relevant to human health. This study will be the first to examine the influence of TCDD on the human hs1,2 enhancer in a human cell line model and should contribute to understanding the etiology of diseases related to the hs1,2 enhancer. Moreover, this study may elucidate the source of the divergence in transcriptional regulation seen between the human and mouse hs1,2 enhancers which is highly significant because if major differences in regulation between human and mouse exist, these differences need to be taken into consideration when interpreting future toxicological and mechanistic studies specific to Ig expression using mouse models.

MATERIALS AND METHODS

Chemicals and reagents

TCDD in 100% DMSO was purchased from AccuStandard Inc. (New Haven, CT). Dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS, *Escherichia coli*) were purchased from Sigma Aldrich (St Louis, MO). CpG was purchased from the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University (East Lansing, MI) and R848 was purchased from Alexis Biochemicals (San Diego, CA).

Cell line models

The IM-9 cell line was purchased from American Tissue and American Type Culture Collection (Rockville, MD). The IM-9 cell line is an Epstein-Bar Virus-transformed B lymphoblastoid cell line which was isolated from a human Caucasian female patient with multiple myeloma. The IM-9 cell line was characterized by Fahey, Sox, and Buell (1971). IM-9 cells were grown in RPMI 1640 media (Mediatech, Inc., Manassas, VA) supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 10 mM HEPES, 20 mM sodium bicarbonate (Sigma Aldrich), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin (Fisher

Scientific, Hanover Park, IL), and 50 μ M 2-mercaptoethanol (Pierce, Rockford, IL). The CH12.LX cell line was generously donated by Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). The source of the CH12.LX cell line was the murine CH12 B-cell lymphoma, which was characterized by Bishop and Haughton (1996). CH12.LX cells were grown under the same media conditions as the IM-9 cells except with 13.5 mM HEPES and 0.1 mM nonessential amino acids and no supplemental L-glutamine or sodium bicarbonate was added. All cells were maintained at 37 °C in a 5% CO₂ atmosphere.

Western blot

Preceding western blot analysis 50 mL of IM-9 cells in triplicate were left untreated or treated with vehicle (0.019% DMSO) or TCDD (30 nM) and incubated for 24 hr at 37 °C in a 5% CO₂ atmosphere. Following incubation, the cells were centrifuged at 250 x g for 5 min at 4 °C. The remaining pellet of cells was incubated for at least 1 h at -80 °C with mild lysis buffer (NP-40, 1M NaCl, 0.1M NaPO₄, 0.5M EDTA) containing freshly added Complete Mini protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) to lyse the cells. After samples were thawed on ice and cleared of debris by centrifugation, total protein per sample was determined using Bradford analysis (Bio-Rad Laboratories, Hercules, CA). One hundred μ g of total protein per sample was then analyzed for the presence of the AhR by Western blot analysis. Briefly, cell lysates were resolved by electrophoreses with a denaturing 10% polyacrylamide SDS-PAGE (National

Diagnostics, Manville, NJ) in SDS electrophoresis running buffer (5 mM TRIS, 192 mM Glycine, 3.4 mM SDS) at 125V for 1.5 h. The proteins were transferred in Tris/borate/EDTA buffer (TBE) (890 mM Tris base, 890 mM boric acid, 0.5M EDTA) to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were then blocked overnight at 4°C with 5% nonfat dairy milk (Kroger, Cincinnati, OH) and 0.05% Tween (Sigma) in TRIS buffered saline. After blocking, the membrane was washed four times with 1x TBS for 5 min on a rocker plate (GyroTwister, Woodbridge, NJ). The membrane was incubated in a solution of 1:1000 primary antibody against the AhR (Abcam, Cambridge, MA), 3% bovine serum albumin (BSA) (Calbiochem, La Jolla, CA), and 0.05% Tween in TBS for 1 hr. The membrane was again washed four times for 5 min each with 1x TBS on a rocker plate. A 1:5000 solution of goat anti-mouse secondary (Abcam) antibody conjugated with horseradish peroxidase, 3% BSA, and 0.05% Tween in TBS was applied to the blot for 1 hr. The blot was rinsed 4 times for 5min each with 1x TBS on a rocker plate. Supersignal western pico chemiluminescence substrate (ECL) (Thermo Scientific, Rockford, IL) was used to visualize the protein on a Fuji Las 3000 imager (FujiFilm Corporation, Tokyo, Japan).

RNA isolation

CYP1A1 RNA transcripts were amplified by RT-PCR. Briefly, 1mL of IM-9 cells, in triplicate, were left untreated or treated with vehicle (0.019% DMSO) or TCDD (30nM) then incubated for 24h at 37°C in a 5% CO₂ atmosphere. Following the incubation period, samples were centrifuged at 2000 rpm and pellets were re-suspended

in 100 μ L of Tri-Reagent (Sigma Life Science, St Louis, MO) then stored at -80°C.

Samples were thawed at room temperature then centrifuged at 12,000 x g for 10min. The aqueous layer was removed and mixed with 10 μ L BCP (Sigma-Aldrich) then loaded into Phase Lock Gel tubes (5PRIME, Gaithersburg, MD) and vortexed. Tubes were incubated for 10min at room temperature then centrifuged at 12,000 x g and 4°C for 5min to clear the Tri reagent phenol from the samples. The clear aqueous layer was removed and combined with 100 μ L isopropanol (Pharmco-Aaper and Commercial Alcohols, Brookfield CT) and incubated for 10min at room temperature. Samples were centrifuged for 8min at 12,000 x g at 4°C then pellets were washed with 75% ethanol (ACROS Organics, Geel, Belgium). Pellets were dried for 15min then re-suspended in 25 μ L of distilled water and dissolved by rocking motion for 30 min then stored at -80°C.

Reverse Transcriptase and Real-time Polymerase Chain Reaction (RT-PCR)

RNA isolated above was reverse transcribed into cDNA using RT-PCR. RNA samples were measured for total RNA using the NanoDrop (NanoDrop Technologies, Wilmington, DE). Each sample was normalized to a concentration of 1 μ g/19.25 μ L. A master mix solution was made using the Taqman RT-PCR kit (Applied Biosystems, Branchburg, NJ). Briefly, 19.25 μ L of RNA sample combined with 5 μ L Taqman RT buffer, 11 μ L 25mM MgCl₂, 10 μ L dNTPs, 2.5 random hexamers, 1 μ L RNase inhibitor, and 1.25 μ L Reverse Transcriptase were reverse transcribed using a Thermo cycler

(Eppendorf, Hauppauge, NY) for 25°C for 10min, 48°C for 30min, 95°C for 5min, then a 4°C hold. The resultant cDNA samples were then stored at -80°C.

Amplification of Cyp1a1 transcripts from the cDNA above was measured by Real Time Polymerase Chain Reaction. Briefly, Cyp1a1 and B-actin primers were generated using Primer3 Software (<http://fokker.wi.mit.edu/primer3/input.htm>). Ten pmol of forward and reverse Cyp1a1 primers (FP: CCTCTTTGGAGCTGGGTTTG; RP:GCTGTGGGGGATGGTGAA) and 2.5µL of 1:10 diluted DNA, were added to 2x SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Then, SYBR Green incorporation was measured for 40 cycles by the 7500 real-time PCR system (Applied Biosystems, Warrington, UK). Amplified transcripts of β-actin, a housekeeping gene (FP:TCACCCACACTGGGCCCATCTACGA; RP;CAGCGGAACCGCTCATTGCCATGG) were measured in a separate tube as a positive control. Fold induction was measured relative to the vehicle samples.

Enzyme Linked Immunosorbent Assay (ELISA)

IM-9 cells were grown to stationary phase ($1.2 - 1.4 \times 10^6$ C/mL) then cut back to log phase, $6.0 \times 10^4 - 5.0 \times 10^5$ C/mL, based on total treatment time. One milliliter samples of IM-9 cells, in triplicate, were left untreated or treated with vehicle (0.019% DMSO), R848 (0, 10, 100, or 1000 nM), CpG (0.1, 0.5, 1, 3, 5, or 6 µM) or TCDD (30nM) then incubated at 37°C in a 5% CO₂ atmosphere for 4 – 96 hr. Twenty microliters of each sample was then diluted 1:1 with Trypan Blue (Beckman Coulter, Brea,

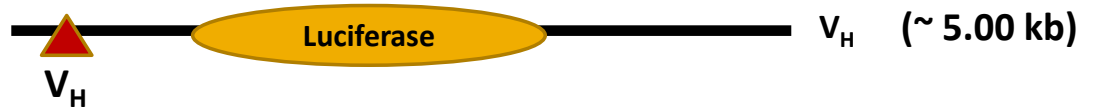
California) and counted by hemocytometer (AO Scientific Instruments, Buffalo, NY). The remaining samples were centrifuged at 250 x g and pellets were re-suspended in 50 μ L mild lysis buffer containing complete mini (protease inhibitor). Supernatants were analyzed by sandwich ELISA for IgG, as previously described (Sulentic et al., 1998). Briefly, 100 μ L of supernatant or IgG standard (human, Bethyl Laboratories, Montgomery, TX) was loaded to a 96-well plate coated with 1:2500 goat-anti-human capture antibody (Southern Biotech, Birmingham, AL), diluted in 0.1% sodium bicarbonate then incubated for 1.5 hr. Following the incubation period, the plate was washed 3x with 0.05% Tween in phosphate buffered saline (PBS) and 4x H₂O by a plate washer. Horseradish peroxidase anti-human capture antibody (Bethyl Laboratories) diluted 1:10000 in a solution of 3% BSA, 0.05% Tween, and PBS was loaded to the plate and incubated for 1.5 hr. Excess antibody was washed 3x with 0.05% Tween in PBS and 4x with H₂O by a plate washer. Colorimetric detection of IgG captured on the plate was performed by adding 100 μ L of ABTS substrate (2,29-azinobis(3-ethylbenz thiazoline-sulfonic acid)) (Roche Diagnostics, Indiana, IN) and measuring the absorbance using the kinetic mode (1 min intervals for 60 min) using a Spectramax plus 384 automated microplate reader with a 405 nm filter (Molecular Devices, Sunnyvale, CA). The concentration of IgG in each sample was calculated from a standard curve of known concentrations of human IgG using the SOFTmax PRO analysis software (Molecular Devices).

Reporter plasmids

Human luciferase reporters were kindly provided by Dr. Michel Cogné, (Laboratoire d'Immunologic, Limoges, France). The reporters consisted of an enhancerless variable heavy chain promoter (V_H) 5' to the luciferase gene or the V_H promoter and the luciferase gene upstream of the human polymorphic hs1,2 enhancer consisting of either one ($\alpha 1A$), two ($\alpha 1B$), or three ($\alpha 1C$) 53bp repeats, as described previously (Denizot et al. 2001) (Fig. 7). All plasmids were constructed using a pGL3 basic luciferase reporter construct, (Promega, Madison, WI). A BSAP binding site added to the $\alpha 1A$ human hs1,2 enhancer reporter ($\alpha 1A$ +BSAP) was previously prepared by our laboratory using site-directed mutagenesis according to QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotide primers were designed to add a BSAP binding motif (TS- GTGGTCCCAGTGTCAGCCCTGGGGTGTTGAGCC-ACCCATCCTTGCCCTAACCCAAGTGGGCCT; BS-AAGCCCACTTGGGTTAGGG-CAAGGATGGGTGGCTCAACACCCCAGGGCTGACACTGGGACCAC) using the QuikChange Primer Design Program (<http://www.stratagene.com/sdmdesigner>). Briefly, 10 ng of the $\alpha 1A$ human hs1,2 reporter was combined with 5 μ L 10x reaction buffer, 125 ng of each oligonucleotide primer, 1 μ L of dNTP mix, 3 μ L of QuikSolution, and 1 μ L of *PfuTurbo* DNA polymerase to a final volume of 50 μ L in thin-walled PCR tubes. Samples were heated to 95 °C for 1 min then cycled 18 times, at 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 6 min per cycle (1 min/kb). A 7 min final extension step occurred at 68 °C after 18 cycles. Next, the parental DNA template was digested with Dpn I. The remaining DNA plasmid was transformed into XL10-Gold Ultracompetent cells, then

isolated and the desired addition validated by sequencing (Retrogen, Inc., San Diego, CA).

V_H promoter luciferase reporter (control)



Human polymorphic hs1,2 enhancer luciferase reporters

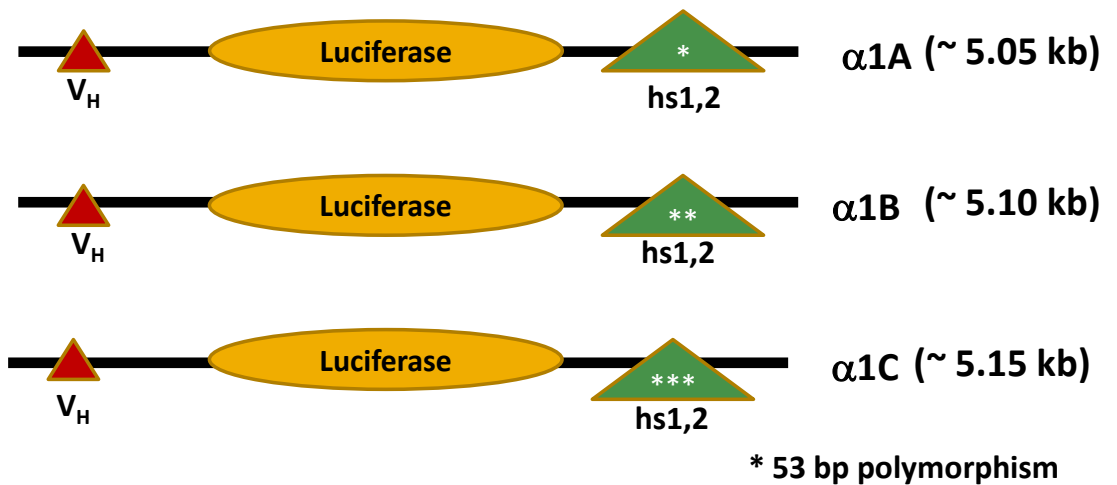


Figure 7. Human hs1,2 enhancer luciferase reporter constructs. Each reporter plasmid was made using a pGL3 basic luciferase construct and contained a variable heavy chain (V_H) promoter. The number of 53bp repeats in each allele of the polymorphic human hs1,2 enhancer is denoted by asterisk “*.”

Transient transfections

IM-9 cells were grown up to their stationary phase concentration ($1.2 - 1.4 \times 10^6$ C/mL) then 1.0×10^7 C were suspended in 200 μ L of culture media with 10 μ g of reporter and transferred to a 2 mm gap electroporation cuvette (Molecular Bioproducts, San Diego, CA). Cells were electroporated using an electro cell manipulator (ECM 630, BTX, San Diego, CA) at a voltage, capacitance, and resistance of 150 V, 1700 μ F, and 75 Ω , respectively. Multiple transfections per plasmid were pooled and divided into treatment groups at a concentration of 1×10^6 C/mL. CH12.LX cells were grown up to log phase (5×10^5 C/mL) then 1.0×10^7 C were electroporated as described above at a voltage, capacitance, and resistance of 250 V, 150 μ F, and 75 Ω , respectively. Multiple transfections per plasmid were pooled and divided into treatment groups at a concentration of 5×10^5 C/mL. Immediately following transfection, unstimulated IM-9 cells were treated with vehicle (0.019% or 0.1% DMSO) or TCDD, or were co-treated with TCDD and CpG and/or R848. Unstimulated or LPS-stimulated CH12.LX cells were treated with (0.019% DMSO) or TCDD (30nM), or were co-treated with TCDD and LPS. Treated IM-9 and CH12.LX cells were aliquoted in triplicate into 12-well plates for a 24h incubation period. Cells were then lysed with 1x reporter lysis buffer (Promega) and frozen at -80°C for at least 1 hr.

Luciferase Assay

To measure luciferase activity, samples were thawed on ice, centrifuged at 14,000 rpm for 5 min. Then twenty microliters of sample supernatant was added to 100 μ L luciferase assay reagent (Promega). Luciferase activity (i.e. luminescence) was measured using a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) and was represented as relative light units.

Traditionally transfection efficiency is determined by co-transfecting a control plasmid such as β -gal. However, common stimulants, such as LPS reportedly activate the promoter and enhancers of control plasmids, making precise standardization of the activity of the control plasmid difficult to determine. Therefore, transfection efficiency was measured by quantitative real-time PCR. DNA was isolated from naïve samples that had been incubated for 2h following transfection using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). Purified DNA was diluted 10-fold and then analyzed by real-time PCR for the luciferase gene. Briefly, primers specific for the luciferase gene encoded with the pGL3 luciferase vector series, were previously generated by our laboratory using Primer3 Software. Next, 10pmol of forward and reverse primers and 2 μ L of diluted purified DNA, were added to 1x SYBR Green PCR Master Mix (Applied Biosystems). Then, SYBR Green incorporation was measured for 40 cycles by the 7500 real time PCR system (Applied Biosystems). A standard curve of known quantities of plasmid DNA was utilized to measure the concentration of plasmid DNA. The total amount of plasmid (ng of plasmid) was calculated by multiplying the concentration of plasmid DNA (ng of plasmid/ μ L), the volume of DNA added (2 μ L),

and the fold dilution (10). The plasmid number per cell was calculated using the following equation: [(nanograms of plasmid) x (molecules of plasmid / nanograms of plasmid) / cell number], as previously described (Sulentic et al., 2004).

Statistical Analysis of Data

The mean \pm SE (n=3) was calculated for each treatment group within each experiment. Significance between treatment groups of real-time PCR studies was determined by a 1-way ANOVA followed by a Dunnett's two-tailed *t* test. Significance between treatment groups and time points in ELISA studies was determined by 2-way ANOVA followed by a Bonferroni's two-tailed *t* test. In luciferase assays, significance between treatment groups of 1 plasmid was determined by 1-way ANOVA followed by a Dunnett's two-tailed *t* test. Significance between multiple plasmids was determined by 2-way ANOVA followed by a Bonferroni's two-tailed *t* test. TCDD-induced fold activation in luciferase studies was calculated by dividing the mean luciferase activity of TCDD and/or stimulant-treated samples by the mean luciferase activity of the appropriate vehicle. Mean fold change for TCDD activation \pm SE was determined by analyzing eleven separate experiments. Luciferase activity relative to transfection efficiency was calculated by dividing the mean plasmid number of the V_H reporter by the mean plasmid number of the appropriate hs1,2 reporter. The quotient was then multiplied to the luciferase activity results for each treatment group for the appropriate hs1,2 reporter.

RESULTS

Characterization of IM-9 cells

Our lab has utilized the mouse CH12.LX cell line extensively to study the role of the AhR/DRE in the inhibitory effect of TCDD on antibody secretion and Ig expression (Sulentic et al., 1998; Sulentic et al., 2000) as well as the TCDD-induced inhibition of 3'IgHRR activation (Sulentic et al., 2004a; Sulentic et al., 2004b). In order to translate our mouse findings into relevant human application it is necessary to utilize a human B-cell line model in which to study the effects of TCDD on the human 3'IgHRR. We decided to characterize the human lymphoblastoid IM-9 cell line as a model for TCDD toxicology studies because: 1) it demonstrated TCDD-induced binding of the AhR to an oligomer containing a DRE site from the Cyp1a1 gene (Masten and Shiverick, 1996), a metabolic gene of which expression is a hallmark of a functional AhR pathway; 2) our preliminary EMSA analysis demonstrated TCDD-induced binding of the AhR from IM-9 cells to an oligomer containing the DRE-like site from the human hs1,2 enhancer, also suggesting a functional AhR pathway (data not shown); 3) we successfully transiently expressed a luciferase reporter regulated by the human hs1,2 enhancer in IM-9 cells, which supported the use of IM-9 cells in studies of transcriptional activation of the human hs1,2 enhancer. Collectively, our preliminary results supported the characterization of the IM-9 cell line for the use as a model for determining the molecular mechanisms behind TCDD-induced modulation of the 3'IgHRR.

IM-9 cells express the AhR: The expression of a functional AhR is a necessity for our studies, as the mechanism for TCDD-induced effects is thought to be mediated by the AhR. By Western blot analysis we confirmed AhR expression in unstimulated IM-9 whole cell lysates (Fig. 8). Since unstimulated human primary B cells were reported to have low expression of AhR (Allan and Sherr, 2005), we loaded 100 µg of IM-9 protein versus 50µg of CH12.LX protein. Accordingly, our results confirmed the expression of AhR in IM-9 cells. Interestingly, although twice as much total protein from IM-9 cells versus CH12.LX cells was loaded to the blot, the levels of AhR expression from the two cell lines appeared to be similar. Notably, whereas the size of mouse AhR has been reported to be 96 kDa, human AhR has been reported to be 110 kDa, which corresponds with our results. In previous studies cellular activation resulted in an increase in AhR and CYP1A1 expression in mouse primary splenocytes and isolated primary B cells (Crawford et al., 1997; Marcus et al., 1998). In addition, activated mouse primary B cells treated with TCDD demonstrated an increase in AhR binding to a DRE oligomer versus cells that were not activated in EMSA/Western studies (Crawford et al., 1997). These results suggest that increased AhR expression due to activation of mouse primary B cells may result in an increase in B cell sensitivity to TCDD and activation of the AhR pathway. In our previous experiments, we activated AhR expression in mouse CH12.LX cells with LPS, a toll-like receptor 4 (TLR4) ligand and B-cell stimulant (Sulentic et al., 1998). However, it has been reported that while LPS is not an efficient activator of human B cells, CpG, a TLR9 stimulant, effectively activates human B cells (Bauer et al., 2001). Likewise Allan and Sherr (2005) found an upregulation of AhR expression and ligand-independent activation in CpG-activated primary human B-cells. Thus, we

attempted to upregulate AhR expression by activating the IM-9 cells with CpG. We found that only 24 h of CpG treatment may have slightly activated the AhR in IM-9 cells. However, expression of the AhR in IM-9 cells was not appreciably upregulated as a result of CpG treatment (Fig. 8), which suggests that CpG may not be an effective activator of IM-9 cells, particularly based on later studies demonstrating a lack of IM-9 activation with CpG (see below). Nonetheless, upregulation AhR was not a necessity for our studies, as the basal expression of AhR was clearly demonstrated, warranting further characterization of the IM-9 cell line (Fig. 8)

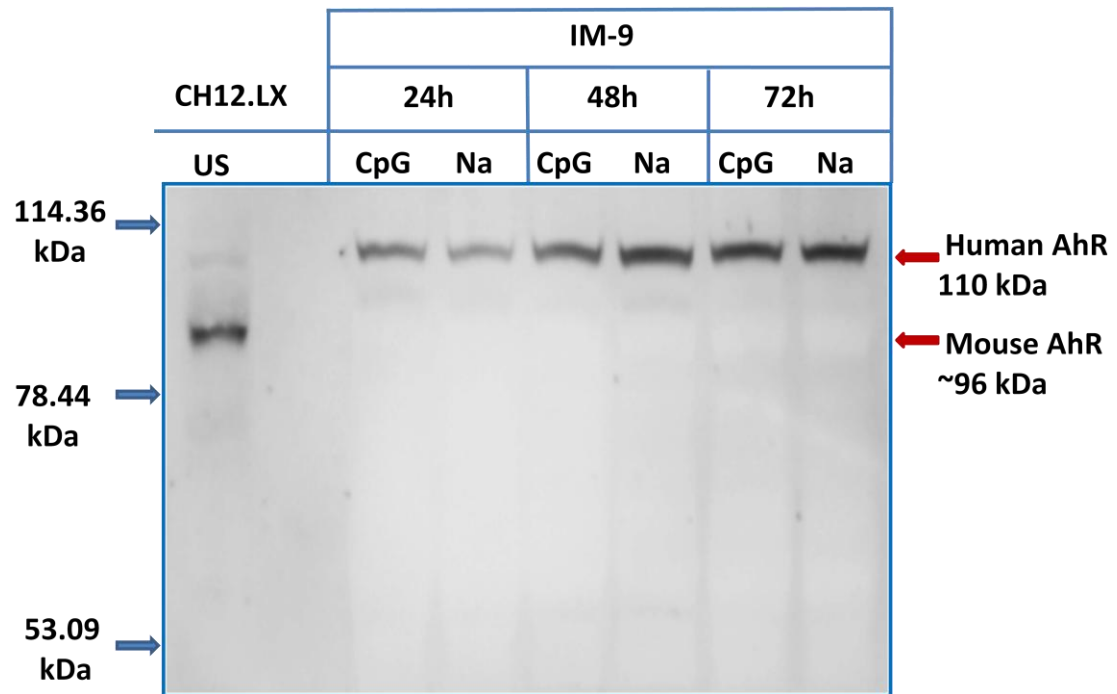


Figure 8. CpG treatment does not appreciably increase AhR expression in human IM-9 cells. Whole cell lysate (100 µg) from naïve (Na) IM-9 cells (5×10^5 C/mL) or cells treated with CpG (1 µM) for 24, 48, or 72 h was loaded into lanes 2-7, resolved on a 10% SDS-PAGE gel, and probed with mouse anti-AhR antibody (0.1 µg/mL). Lysate (50 µg) from naïve CH12.LX cells (5×10^5 C/mL) was loaded into lane 1 as a positive control.

IM-9 cells have a functional AhR signaling pathway: Since TCDD-induced effects are thought to be mediated through the AhR pathway, a functional AhR pathway in IM-9 cells is essential for our mechanistic studies. As stated above, several past mechanistic studies have established induction of the CYP1A1 gene, a metabolic gene, as the hallmark of a functional AhR pathway (see Ma, 2001 for review). Therefore, we chose to utilize real-time RT-PCR to quantify RNA transcripts for the CYP1A1 gene in naïve or TCDD-treated IM-9 cells. We found a marked induction of CYP1A1 in the TCDD-treated cells which suggest that IM-9 cells have a functional AhR pathway (Fig. 9).

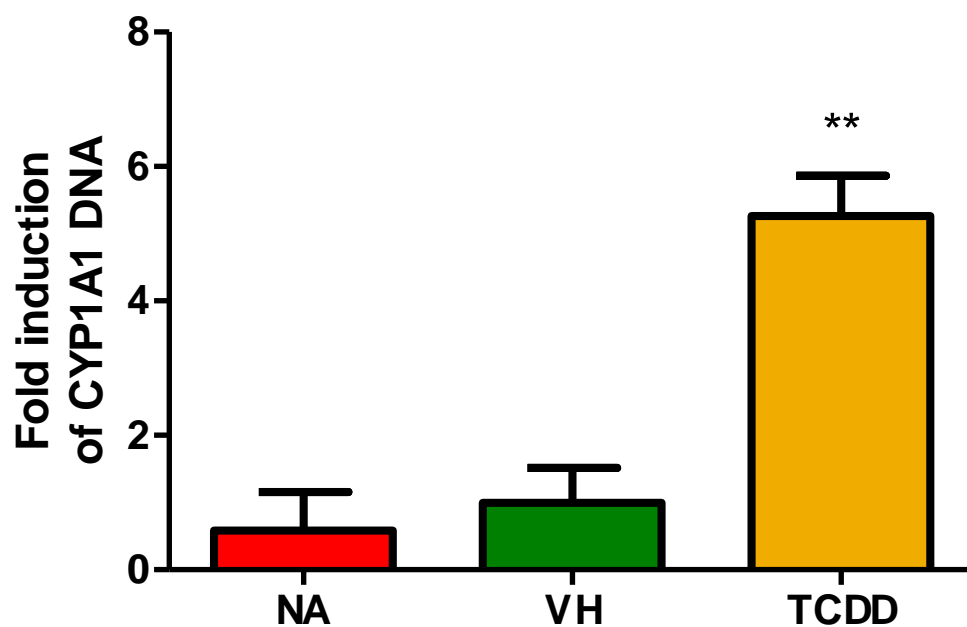


Figure 9. TCDD induces CYP1A1 induction. Reverse transcribed cDNA from untreated IM-9 cells or cells treated for 24h with vehicle (0.019% DMSO) or 30 nM TCDD was amplified by real-time PCR with β -actin as an internal control. Fold induction was relative to NA.

IM-9 cells express basal IgG: Previous experiments in our laboratory support the theory that TCDD inhibits antibody secretion through the AhR pathway in the mouse CH12.LX cell line (Sulentic et al., 1998; Sulentic et al., 2000). Therefore, we hypothesize that modulation of Ig secretion is a toxicological endpoint of TCDD induction of the AhR pathway in a human cell line. In order to determine if IM-9 cells are a viable model for studying the TCDD-induced effects on Ig secretion, we chose to characterize basal Ig secretion in IM-9 cells. Importantly, a prerequisite for determining antibody secretion was to develop the most effective culturing conditions for the IM-9 cell line by analyzing proliferation patterns corresponding with basal Ig secretion. A complex proliferation and basal Ig secretion pattern in the IM-9 cell line was reported by Fahey, Buell and Sox (1971). Since cell lines are known to change their phenotype over time, likely due to various reasons including different culturing practices, our studies focused first on verifying optimum culturing conditions for activation of Ig expression. In correlation with the Fahey, 1971 study, we found that in order to see significant basal IgG secretion, IM-9 cells had to be grown up to a stationary growth phase (cell concentration $\geq 1.2 \times 10^6$ C/mL) then reduced in concentration to obtain a log growth phase (cell concentration between 2.5×10^4 and 5.0×10^5 C/mL). Then this cycle of concentration growth and reduction was to be repeated once. After the second reduction in concentration to obtain a log growth phase, basal Ig was expressed and could be sampled as cells proliferated back up to a stationary phase. Using these culturing practices we established that the current IM-9 cell line secretes IgG, however, when normalized to cell number, naïve IM-9 cell supernatant only demonstrated low levels of secreted IgG in the magnitude of ng per 10^5 cells (Fig. 10), whereas the levels of secreted IgG seen in the Fahey study (1971) was in

the magnitude of hundreds of ng per 10^5 cells. Differences in magnitude of basal IgG secretion suggest that the IM-9 cell line may have changed in phenotype or our particular culturing conditions, such as the type and lot of serum, may influence the basal IgG levels. Interestingly, physiological levels of human IgG are g per liter in magnitude. Thus, our levels of IgG secretion in the human IM-9 cell line may not be physiologically significant.

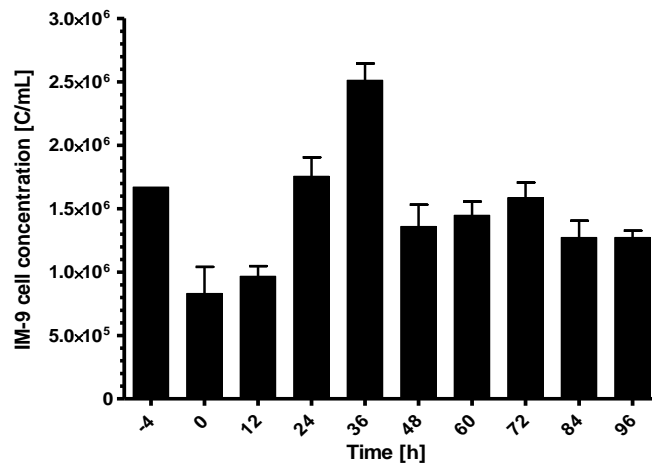
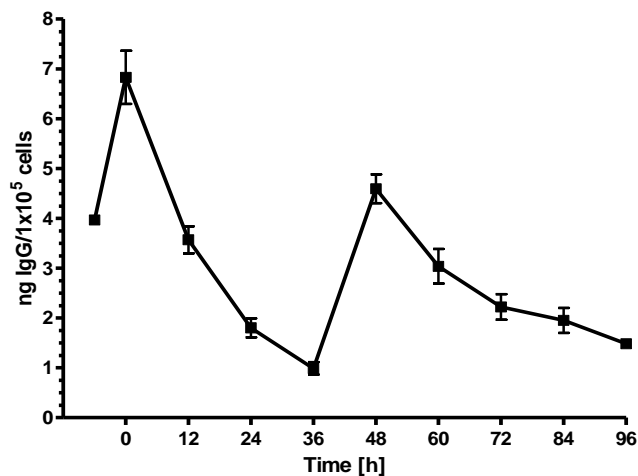
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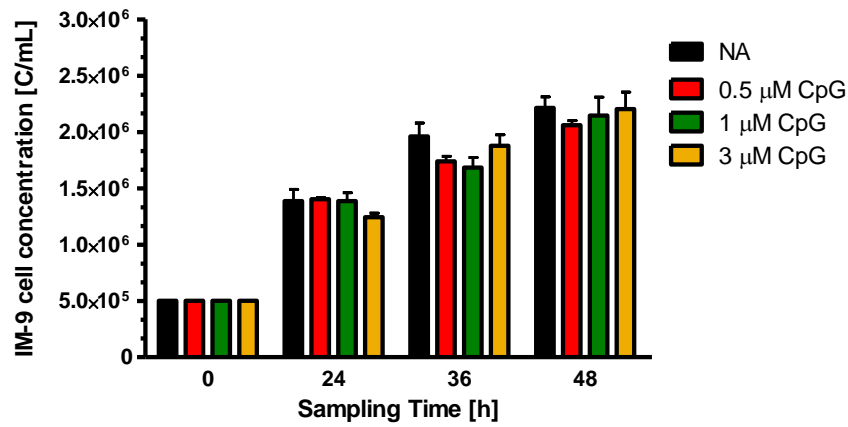
Figure 10. Basal IgG secretion from IM-9 cells normalized to cell number. IM-9 cells were allowed to grow to a stationary concentration (cell concentration $\geq 1.2 \times 10^6$ C/mL) then reduced in concentration to a log concentration (cell concentration between 2.5×10^4 and 5.0×10^5 C/mL) (data not shown). Then cells were again grown to a stationary phase (1.5×10^6 C/mL) then reduced in concentration to a log phase (5.0×10^5 C/mL). A, the first cell concentration was measured by hemocytometer during the second stationary phase just prior to the second concentration reduction to 5×10^5 C/mL (-4 h). Then, following a 4 h recovery period, samples were collected and cell concentration was measured in 12 h intervals for 96 h. B, secreted IgG concentration was measured by ELISA.

IgG expression in IM-9 cells is not significantly activated by CpG or R848: We

previously demonstrated in mouse CH12.LX cells that IgM expression activated by LPS-stimulation was inhibited by TCDD (Sulentic et al., 1998). Since in CH12.LX cells TCDD-induced inhibition of Ig required cellular activation we attempted to identify a B-cell activator for our human IM-9 cells. As previously stated, CpG has been shown to activate human B cells (Bauer et al., 2001). In our earlier studies we found that CpG did not appreciably stimulate upregulation of the AhR. However, there was still the possibility that CpG would stimulate IgG expression. Thus, we tested CpG as a stimulant of IgG expression in IM-9 cells and found a modest activation of IgG at 36 h by 0.5 μ M CpG (Fig. 11), however higher concentrations (1 and 3 μ M) of CpG significantly inhibited background IgG expression (fig 11). Given that the CpG activation we saw was not significant, we considered testing R848 as a stimulant. R848 has been reported as a TLR7 agonist that mimics CD40L activation (Bishop et al., 2001). CD40L is primarily expressed on activated T cells and was demonstrated by Allan and Sherr (2005), to be a promising but expensive stimulant of primary human B cells. Thus, we attempted to stimulate IM-9 cells with R848, an affordable substitute to recombinant CD40L. We found little or no significant activation in IgG concentration relative to vehicle control (0.1% DMSO) in IM-9 cells treated with 10-1000 ng/mL R848 for 36-96 h. However, our 24 h samples showed variably showed no effect or inhibition in IM-9 cells treated with 100 ng/mL R848 ($p < 0.05$). (Fig. 12). Although, IM-9 cells have been reported to be high IgG-secreting lymphoblast (Fahey et al., 1971), we did not detect high basal IgG secretion and have yet to find an optimal stimulant for these cells. The appropriate

activation stimuli may be more complex and involve a combination of stimuli including interleukins and CD40L.

A



B

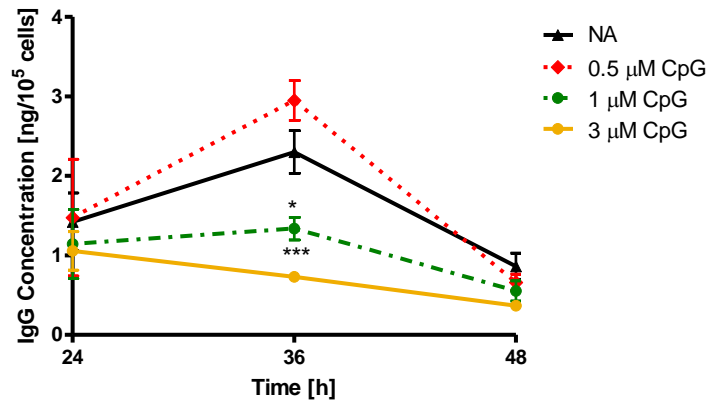


Figure 11. A low concentration of CpG modestly activates IgG secretion in IM-9 cells. IM-9 cells were allowed to grow to a stationary phase concentration (cell concentration $\geq 1.2 \times 10^6$ C/mL) then reduced in concentration to a log phase concentration (cell concentration between 2.5×10^4 and 5.0×10^5 C/mL) (data not shown). Cells were then again grown to a stationary phase concentration and then reduced in concentration to 5.0×10^5 C/mL. Naïve (NA) IM-9 cells and cells treated with 0.5, 1 or 3 μ M CpG were incubated for 24-48h. A, samples were collected and cell concentration was measured by hemocytometer at 24, 36, and 48 h. B, IgG concentration was measured by ELISA. Two-way ANOVA followed by a Bonferroni's two-tailed t test (95% confidence interval) indicates significant inhibition relative to the NA control at a concentration of 1 μ M CpG (*) ($p < 0.05$), and 3 μ M CpG (***) ($p < 0.001$).

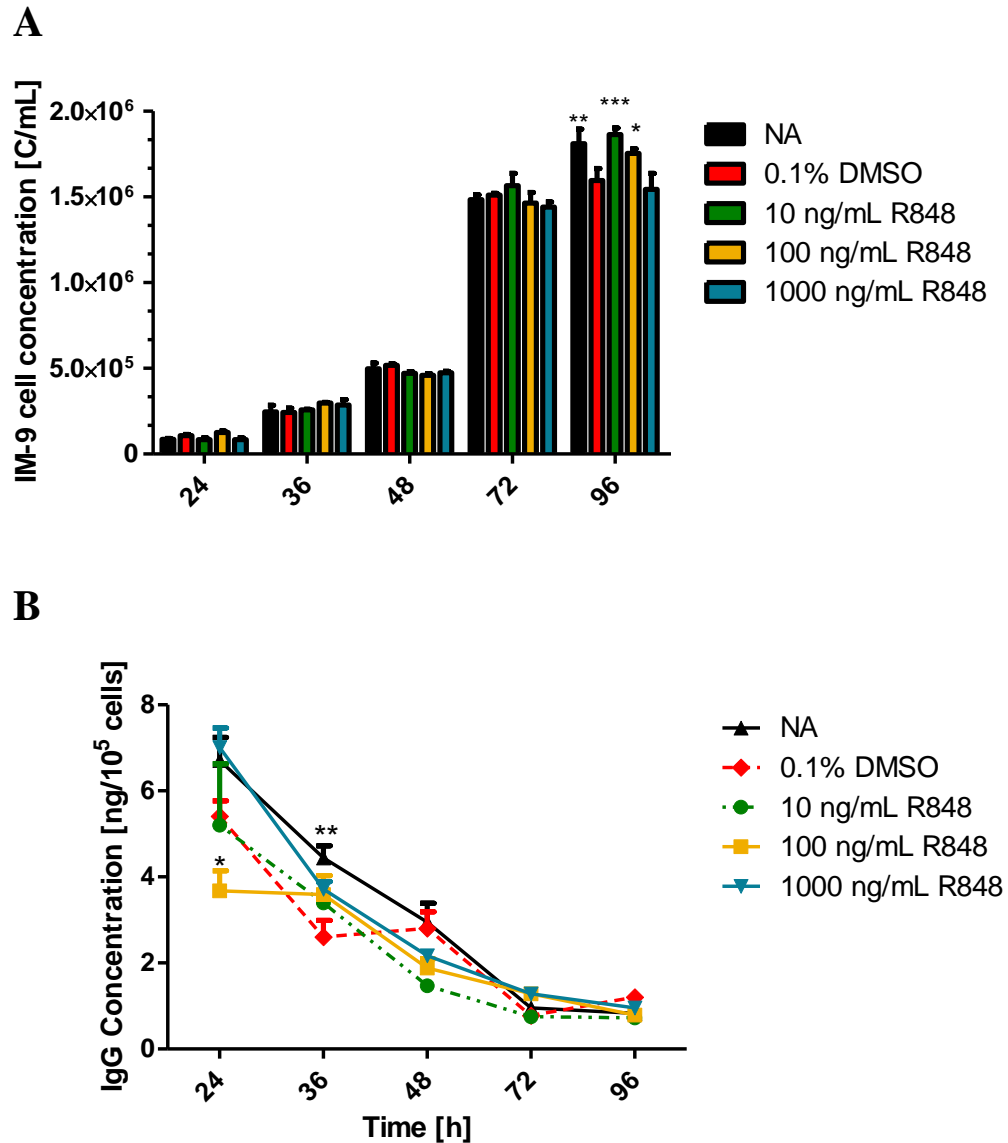


Figure 12. IM-9 cells are not activated by R848. IM-9 cells were allowed to grow to a stationary phase concentration (cell concentration $\geq 1.2 \times 10^6$ C/mL) then reduced in concentration to a log phase concentration (cell concentration between 2.5×10^4 and 5.0×10^5 C/mL) (data not shown). Then, untreated IM-9 cells were again grown to a stationary phase and then reduced in concentration to 3.5×10^4 C/mL. Naïve (NA) IM-9 and cells and cells treated with 0.1% DMSO vehicle, 10, 100, or 1000 ng/mL R848 were incubated for 24-96h. A, Samples were collected and cell concentration was measured by hemocytometer at 24, 36, 48, 72 and 96 h. B, IgG concentration was measured by ELISA. Two-way ANOVA followed by a Bonferroni's two-tailed *t* test (95% confidence interval) indicated significant differences compared to the vehicle control $p < 0.05$ (*), $p < 0.01$ (**).

Transfection of the polymorphic human hs1,2 enhancer into IM-9 cells

Our lab has demonstrated that TCDD induces binding of the mouse AhR to a DRE site within an oligomer from the hs1,2 enhancer region of the 3' IgHRR (Sulentic et al., 2000). We have also shown that TCDD inhibits transcriptional activity of the mouse hs1,2 enhancer (Fernando et al. manuscript in preparation), which suggests that regulation of the mouse hs1,2 enhancer may be a sensitive transcriptional target of TCDD through the AhR signaling pathway. Since the mouse and human hs1,2 enhancers share a 90% core sequence identity (Mills et al. 1997), we propose that the transcriptional regulation of the human hs1,2 enhancer may also be a sensitive transcriptional target of the human AhR pathway. Three allelic polymorphisms of the human hs1,2 enhancer have been reported, $\alpha 1A$, $\alpha 1B$, and $\alpha 1C$, each containing a 1, 2, or 3-53 bp tandem repeats, respectively (Denizot et al., 2001). We previously found that activation of luciferase reporters consisting of the V_H promoter and the $\alpha 1A$, $\alpha 1B$, or the $\alpha 1C$ allele of the hs1,2 enhancer was TCDD concentration-dependent, and cells stimulated with LPS and co-treated with TCDD demonstrated an enhanced activation that was dependent on number of 53 bp repeats in addition to being TCDD and LPS concentration-dependent (Fernando et al., manuscript in preparation). Strikingly, however, the $\alpha 1B$ reporter, the allele that has been associated with several human autoimmune diseases, showed the greatest fold-activation ($p < 0.05$) of the three hs1,2 reporters in LPS-activated and TCDD co-treated CH12.LX cells (Fernando et al., manuscript in preparation). Still, a gap remained in our understanding of the effects of TCDD on transcriptional regulation of the human hs1,2 enhancers in a human cell line. In preliminary experiments we found, in correspondence

with studies in the mouse CH12.LX cell line, the $\alpha 1B$ wild type reporter, transfected into human IM-9 cells demonstrated a TCDD-induced increase in activity. However, the effect of TCDD on the $\alpha 1A$ and $\alpha 1C$ alleles of the hs1,2 enhancer in human cells had not been determined. Accordingly, we conducted transfection studies on all three of the human hs1,2 reporters to evaluate the effects of TCDD in the human IM-9 cell line

Culturing conditions for transfection studies in IM-9 cells: In the above mentioned Ig experiments we determined that IM-9 cells required a complex culturing pattern in order to express Ig. Therefore, to determine ideal culturing conditions, we conducted several studies in which the IM-9 cells were initially grown to lag, log, or stationary phase, transfected with the $\alpha 1B$ reporter, then seeded at lag, log, or stationary phase and treated with TCDD. In addition, previous studies have demonstrated that human AhR has a lower level of endogenous expression and affinity for TCDD than mouse AhR (Harper et al., 1988). Furthermore, we found lower expression of human AhR versus mouse AhR in IM-9 and CH12.LX cells, respectively in our earlier Western Blot studies. Therefore, we initially tested both a standard concentration (30nM, for our laboratory) and a high concentration (100 nM) of TCDD in our culturing condition studies because low AhR expression may be associated with lowered sensitivity to TCDD, as has been suggested in other studies (Harper et al., 1998; Ema et al., 1994). We found a significant TCDD-induced activation in the log-stationary (i.e., grown to log phase then transfected and reseeded at a stationary phase concentration), and stationary-stationary (i.e., grown to stationary phase then transfected and reseeded at a stationary phase concentration) culture conditions with both 30nM and 100nM concentrations of TCDD (Fig. 13). Interestingly,

there was a marked increase in overall luciferase activity levels of the log-stationary samples versus the stationary-stationary samples (Fig. 13). In spite of this, we saw slightly higher TCDD-induced fold-activation in stationary-stationary samples versus log-stationary samples treated with 30 nM TCDD (fig 14). When we compared these results to a pilot study in which we treated the α 1C reporter with 30 nM TCDD, we saw a similar trend in fold-activation (fig 14). Consequently, we decided to use stationary-stationary culturing conditions in our transfection studies. We decided to use 30 nM TCDD versus 100 nM TCDD because 30 nM TCDD is closer to common TCDD exposure levels than the 100 nM TCDD and results from additional studies did not show a great difference in fold induction from 30 nM TCDD versus 100 nM TCDD. This corresponds to studies in mouse CH12.LX cells in which TCDD-induced fold-activation seemed to plateau at 30 nM TCDD (Fernando et al., manuscript in preparation).

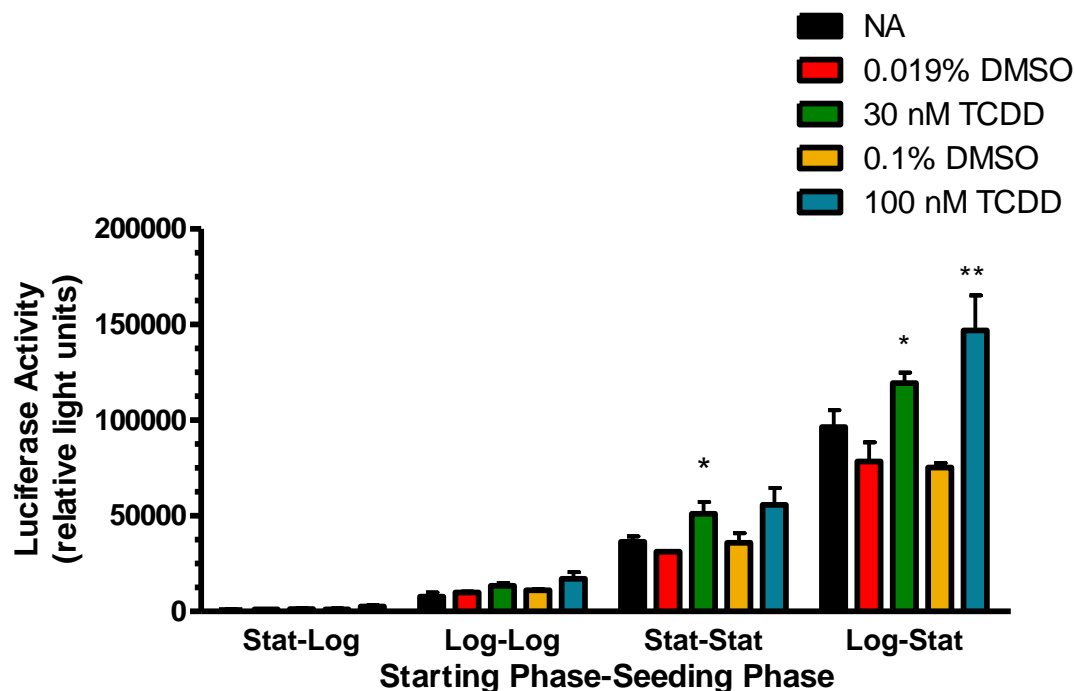


Figure 13. The α 1B reporter is activated by 30 and 100nM TCDD when IM-9 cells are cultured to stationary or log concentrations. IM-9 cells were grown to a stationary phase (cell concentration $\geq 1.2 \times 10^6$ C/mL) or to a log concentration ($\sim 5.0 \times 10^5$ C/mL), transfected with the α 1B luciferase reporter, and then left untreated (NA), or treated with DMSO vehicle or TCDD. The vehicle controls for samples treated with 30 nM or 100 nM TCDD was 0.019% or 0.1% DMSO. Treatment groups with a log phase final seeding concentration were seeded at 5.0×10^5 C/mL. Treatment groups with a stationary phase final seeding concentration were seeded at 1.0×10^6 C/mL and incubated for 24h. Luciferase activity was measured in relative light units. Statistical significance was determined by Two-way ANOVA followed by a Bonferroni's two-tailed *t* test (95% confidence intervals) indicated and is depicted by “*” ($p < 0.05$) or “**” ($p < 0.01$).

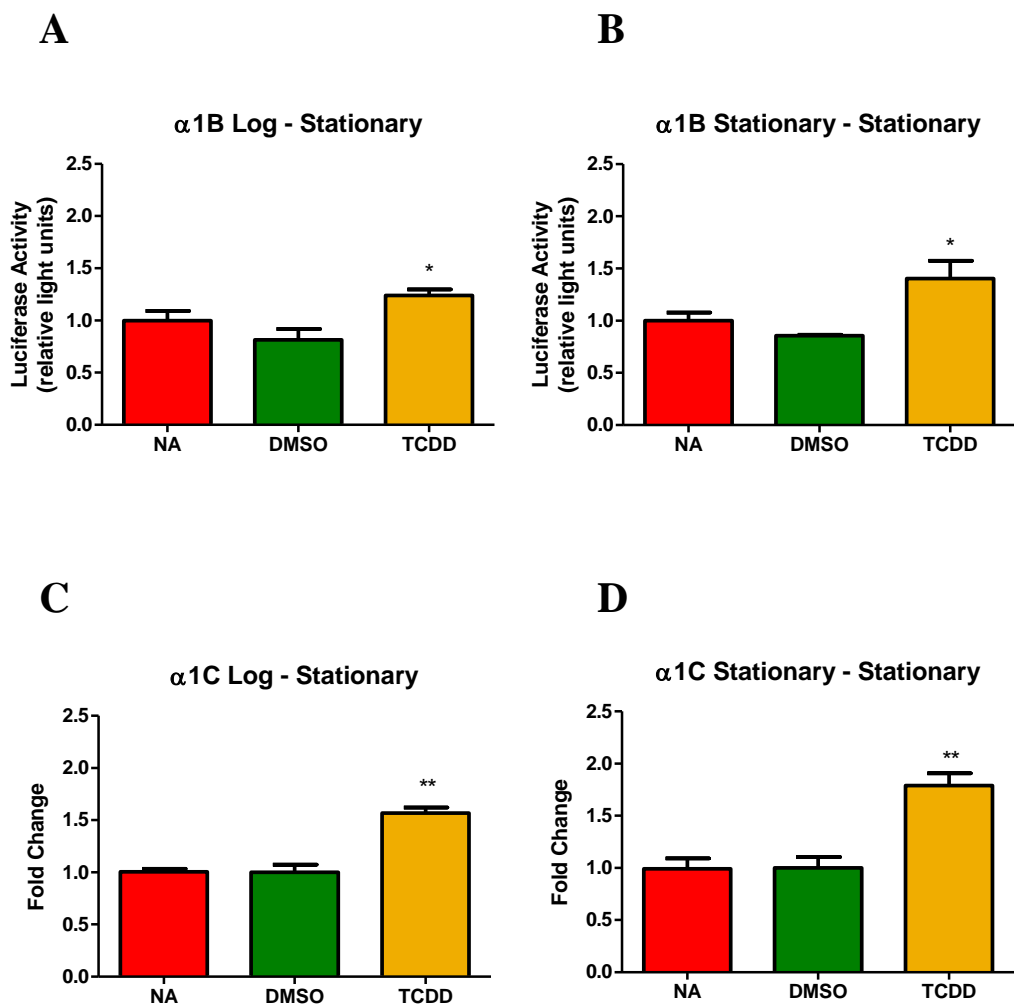


Figure 14. The fold-activation of the $\alpha 1B$ and $\alpha 1C$ reporters is slightly higher in IM-9 cells initially cultured to a stationary phase versus a log phase. IM-9 cells were grown to a stationary phase (cell concentration $\geq 1.2 \times 10^6$ C/mL) or to a log concentration ($\sim 5.0 \times 10^5$ C/mL), then transfected with either the $\alpha 1B$ or $\alpha 1C$ luciferase reporters, and then left untreated (NA), or treated with 0.019% DMSO vehicle (DMSO) or 30 nM TCDD (TCDD). Treatment groups were seeded at 1.0×10^6 C/mL and incubated for 24h. Luciferase activity results were normalized to fold change relative to vehicle control. Statistical significance was determined by one-way ANOVA followed by a Dunnett's two-tailed t test (95% confidence interval) and is depicted by “*” ($p < 0.05$) or “**” ($p < 0.01$).

TCDD activates the human polymorphic hs1,2 enhancer: Once culturing conditions for IM-9 transfection studies were optimized we focused our attention on determining TCDD-induced effects on the $\alpha 1A$, $\alpha 1B$, $\alpha 1C$, alleles of the hs1,2 enhancer as well as a control reporter consisting of the V_H promoter alone. We found that the basal activities of the human hs1,2 reporters were markedly higher in IM-9 cells versus CH12.LX cells (data not shown). In addition we found that in the human IM-9 cell line all three of the hs1,2 reporters were activated by TCDD relative to a vehicle control (0.019% DMSO) (Fig. 15). These results correspond to the activation of each reporter that was seen in CH12.LX cells treated with TCDD. We also compared the averages of TCDD-induced fold-activation of the V_H promoter and hs1,2 reporters relative to vehicle from multiple independent studies in the IM-9 (V_H promoter n=4; $\alpha 1A$ n=9; $\alpha 1B$ n=11; $\alpha 1C$ n= 10) and CH12.LX ($\alpha 1A$ n=4; $\alpha 1B$ n=3; $\alpha 1C$ n=4) cell lines (Table 2). Converse to our basal activity findings, we found a considerably higher increase in the TCDD-induced fold-activation relative to vehicle of the hs1,2 reporters in CH12.LX versus IM-9 cells (Table 2). Moreover, the V_H promoter was only transfected into IM-9 cells in this study, however, the fold-activation of the V_H promoter in IM-9 cells was considerably lower than the fold activation demonstrated in our previous CH12.LX studies (Fernando et al., manuscript in preparation), (data not shown). It is notable, however, that the TCDD-induced increase in fold-activation of the hs1,2 enhancers compared to the V_H promoter in IM-9 cells was not statistically significant (Table 2). However our previous studies also suggest that the V_H promoter may be regulated differently than the hs1,2 reporters after treatment with TCDD and LPS in CH12.LX cells. Nevertheless, supposing the effect of greater TCDD-induced fold-activation of the hs1,2 reporters in CH12.LX cells

versus IM-9 cells is mediated by the AhR, then these findings correspond with previous reports of higher AhR affinity and expression in mouse cells versus human cells.

Furthermore, regardless of the extent of the fold-activation, in both IM-9 and CH12.LX cells the fold-activation of the α 1B reporter was the highest of the human hs1,2 reporters (Table 2), which may be medically relevant to human health based on the association of the α 1B allele of the hs1,2 enhancer to several autoimmune diseases. In total our data demonstrates that the human hs1,2 enhancers are activated by TCDD in a human B-cell line model.

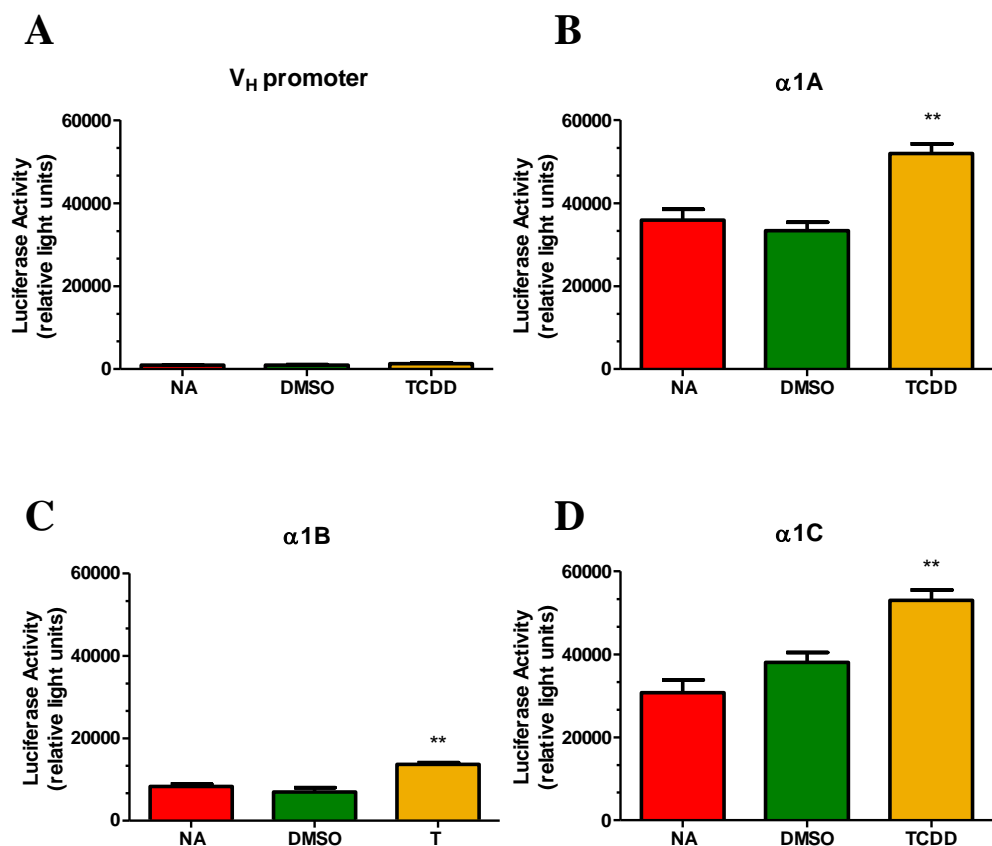


Figure 15. The human *hs1,2* reporters are activated by TCDD in human IM-9 cells. The V_H , $\alpha 1A$, $\alpha 1B$ or $\alpha 1C$ reporters were transfected into IM-9 cells then left untreated (NA) or treated with 0.019% DMSO vehicle control (DMSO) or 30nM TCDD (TCDD) and incubated for 24 h. The results depicted are not from the same study. Luciferase activity results are reported in relative light units and are normalized to the activity of the DMSO vehicle treatment group of each plasmid. Statistical significance was determined by one-way ANOVA followed by a Dunnett's two-tailed t test (95% confidence interval) and is depicted by "***" ($p < 0.01$).

Reporter	CH12.LX (30 nM TCDD)			IM-9 (30 nM TCDD)		
	Fold-activation	±SEM	p-value	Fold-activation	±SEM	p-value
V _H	†	†	†	1.2	0.058	p>0.05
α1A	1.9	0.313	p>0.05	1.4	0.066	p<0.01
α1B	2.6	0.231	p<0.01	1.6	0.116	p<0.001
α1C	1.8	0.288	p>0.05	1.5	0.078	p<0.001

Table 2. Comparisons of TCDD-induced fold-activation relative to vehicle control of the V_H promoter alone and the *hs1,2* reporters in CH12.LX or IM-9 cells.

Comparison of the average fold-activation induced by 30 nM TCDD relative to the 0.019% DMSO vehicle control from multiple transfection studies of the α1A, α1B, and α1C human reporters in unstimulated CH12.LX cells (α1A n=4; α1B n=3; α1C n=4) versus unstimulated IM-9 cells (α1A n=9; α1B n=11; α1C n= 10). Transfection studies utilizing the V_H reporter alone were not conducted in CH12.LX cells, as symbolized by “†.” Significance was calculated by 1-way ANOVA followed by a Dunnett’s two-tailed *t* test (95% confidence interval).

The human hs1,2 enhancer is not activated by CpG

In our past CH12.LX transfection studies we found a correlation between the number of 53bp repeats of each allele of the human hs1,2 enhancer and a LPS-induced increase in the total activity of the V_H promoter and human hs1,2 reporters (Fernando et al., manuscript in preparation). Furthermore, we found that the human hs1,2 enhancer was synergistically activated by TCDD and LPS co-treatment in CH12.LX cells (Fernando et al., manuscript in preparation). Therefore, we decided to determine if the synergistic activation from stimulation and TCDD treatment is also displayed in human IM-9 cells. Although, we did not see an appreciable level of cellular activation of IM-9 cells treated with CpG in our characterization studies, our preliminary experiments demonstrated a slight CpG-induced activation of transcriptional activity of the human α 1B reporter in IM-9 cells (data not shown). Thus, we employed CpG as a stimulant for our transfection studies. However, our data suggests that the transcriptional activation of the α 1B reporter is not enhanced in IM-9 cells co-treated with CpG and TCDD (Fig. 16).

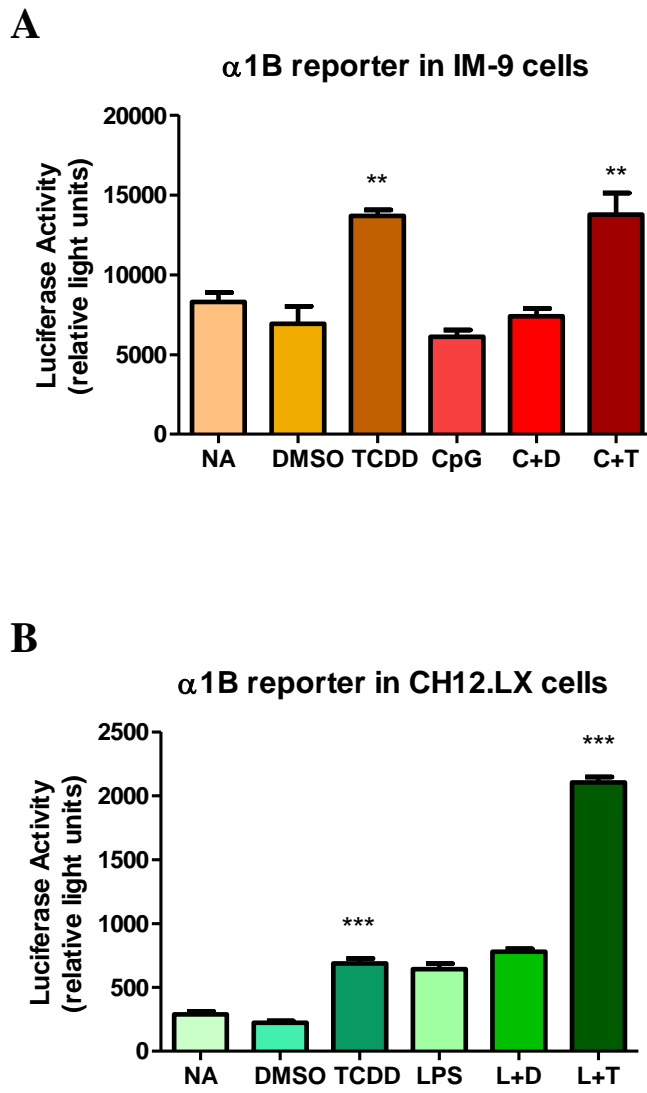


Figure 16. LPS and TCDD co-treatment synergistically activates the α 1B reporter in CH12.LX cells but CpG treatment does not enhance TCDD-induced activation of the α 1B reporter in IM-9 cells. A, Luciferase activity of the α 1B reporter was measured in untreated human IM-9 B cells (NA) or IM-9 cells treated for 24 h with 0.019% DMSO vehicle control (DMSO), 30 nM TCDD (TCDD), 0.5 μ M CpG (CpG), 0.5 μ M CpG + 0.019% DMSO (C+D), or 0.5 μ M CpG + 30 nM TCDD (C+T). B, Luciferase activity of the α 1B reporter was measured in untreated mouse CH12.LX cells (NA) or CH12.LX cells treated for 24 h with 0.019% DMSO vehicle control (DMSO), 30 nM TCDD (TCDD), 1 μ g/mL LPS (L), 1 μ g/mL LPS + 0.019% DMSO (L+D) or 1 μ g/mL + 30 nM TCDD (L+T). Statistical significance was determined by one-way ANOVA followed by a Dunnett's two-tailed *t* test (95% confidence interval) and is depicted by "***" ($p < 0.01$) or "****" ($p < 0.001$).

BSAP decreases TCDD-induced activity of the human hs1,2 enhancer

Mouse models are widely used for toxicological studies based on their overall genetic similarity to humans. As stated previously, the human and mouse hs1,2 enhancers share a 90% core sequence identity (Mills et al. 1997). However, differences between the human and mouse hs1,2 enhancer sequences may impact their transcriptional regulation. Our previous data has shown that TCDD inhibits LPS-stimulated transcriptional activation of a luciferase reporter consisting of the mouse hs1,2 enhancer in the mouse CH12.LX cell line (Fernando et al. manuscript in preparation). Conversely, in CH12.LX cells the human hs1,2 luciferase reporters are activated by TCDD in the absence of stimulation and synergistically activated by a TCDD and LPS co-treatment (Fernando et al. manuscript in preparation). The primary sequence difference between the human and mouse hs1,2 enhancers is the lack in the human hs1,2 sequence of the high affinity BSAP site found in the mouse hs1,2 enhancer sequence (Mills et al. 1997). BSAP is an inhibitor of B-cell differentiation (Nutt et al. 1999). In immature and mature B cells BSAP expression is high, but during the differentiation process BSAP expression is reduced to nil in plasma cells (Reviewed by Nutt et al. 2001). BSAP in conjunction with κ B and Oct motifs exert a concerted repression of the mouse hs1,2 enhancer in mature B cells (Singh and Birshtein 1993, 1995; Michaelson et al., 1996). This is contrary to what is seen in plasma cells where BSAP is not expressed and protein binding to the κ B and Oct motifs within the mouse hs1,2 enhancer exhibits a positive effect (Michaelson 1996). Moreover, Yoo et al. (2003) demonstrated that in CH12.LX cells co-treated with LPS and TCDD, expression of BSAP protein and mRNA was maintained 72h post LPS-activation. The TCDD-induced sustained expression of BSAP could have inhibited the change in role of

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κ B and Oct which positively regulate B cells late in differentiation. This prolonged expression of BSAP and hence, negative regulation by BSAP, was correlated with a decrease in IgH transcripts (Yoo et al. 2003). Together these conclusions led us to the hypothesis that in TCDD-treated cells, the lack of the high affinity BSAP site in the human hs1,2 enhancer when compared to the mouse hs1,2 enhancer could mediate the differences in transcriptional modulation by TCDD. Therefore, oligonucleotide primers were designed to add a BSAP site, identical to the high affinity BSAP site in the mouse hs1,2 enhancer, to the wild type human α 1A reporter (α 1A+BSAP) by site directed mutagenesis (Fernando et al., manuscript in preparation). We then conducted transient transfection experiments using the α 1A and the α 1A+BSAP reporters in IM-9 cells and we found that both reporters were activated by TCDD. We found that, the TCDD-induced fold-activation of the α 1A+BSAP was noticeably lower than the fold-activation of the α 1A reporter (Fig. 17). Therefore, our results suggest that the α 1A+BSAP reporter may be negatively regulated by the added BSAP motif. This is supported by studies from our lab of α 1A+B reporter transfected into the mouse CH12.LX cell line, in which the α 1A+BSAP reporter demonstrated a consistent decrease in total TCDD-induced activation versus the α 1A reporter in LPS induced CH12.LX cells (Ochs et al., manuscript in progress).

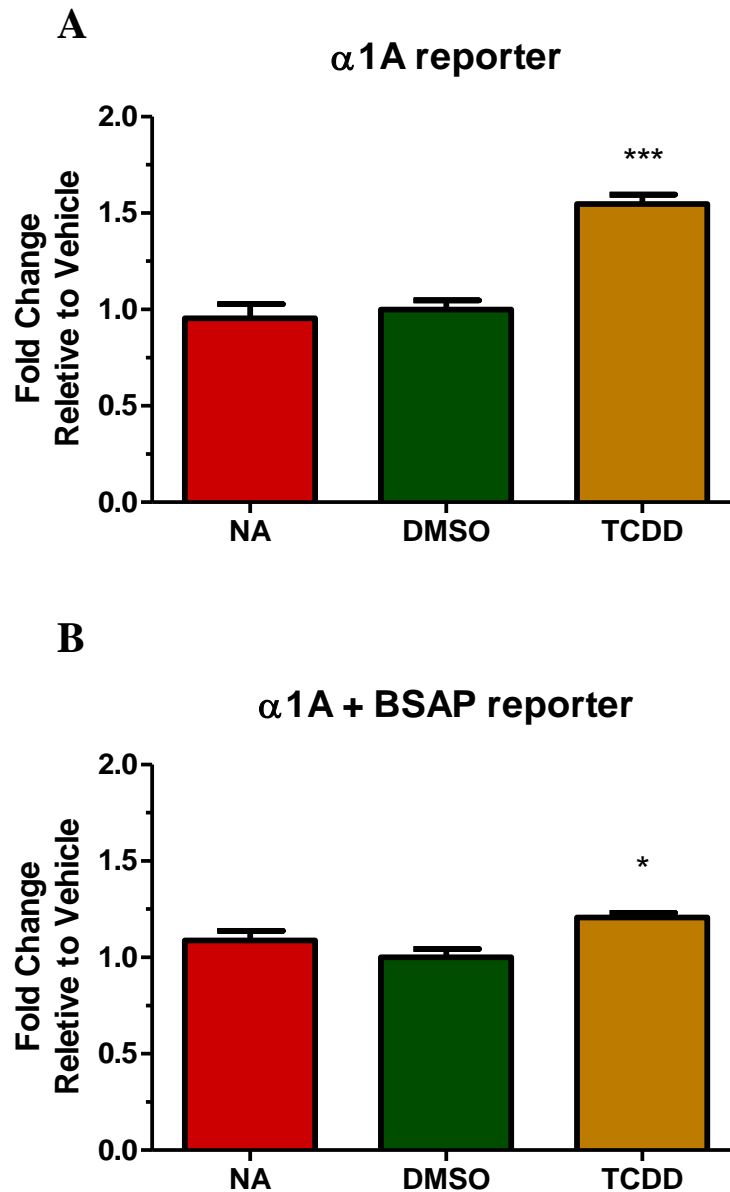


Figure 17. TCDD-induced activation of the human hs1,2 enhancer is inhibited by addition of a BSAP binding motif. IM-9 cells were transfected with the α 1A (A) or α 1A + BSAP (B) reporters then left untreated (NA) or treated with 0.019% DMSO vehicle control (DMSO) or 30 nM TCDD (TCDD) and incubated for 24 h. Luciferase activity was normalized to fold change in activity relative to VH. Statistical significance was determined by one-way ANOVA followed by a Dunnett's two-tailed *t* test (95% confidence interval) and is depicted by “*” ($p < 0.05$) or “***” ($p < 0.001$).

DISCUSSION

The IM-9 cells are an acceptable but not ideal cellular model for mechanistic studies on the effects of TCDD on the 3'IgHRR

The 3'IgHRR has been established as a mediator of VDJ rearrangement (Serwe and Sablitzky, 1993), CSR (Bottaro et al., 1998; Pattersson et al., 1990; Liebersson et al., 1991; Darizvach et al., 1991; Cogné et al., 1994) and Ig expression and secretion (Wabl et al, 1984; Klein et al., 1984) through transcriptional regulation of the IgH gene. Our previous studies suggest that expression and secretion of Ig may be inhibited by TCDD, possibly through the AhR pathway (Sulentic et al., 1998; 2000). We previously hypothesized that the AhR pathway and binding of AhR/ARNT to DRE motifs may be one mechanism behind TCDD-induced modulation of the mouse 3'IgHRR and individual enhancers in activated CH12.LX cells. We have identified DRE-like motifs within the hs4 of the mouse and hs1,2 of the mouse and human 3'IgHRR (Sulentic et al., 2000; Fernando et al., manuscript in preparation). We also found that, in the LPS-activated mouse CH12.LX cell line, TCDD modulates the transcriptional activity of luciferase reporter plasmids consisting of the mouse hs4 enhancer and 3'IgHRR as well as the mouse and human1,2 enhancers of the 3'IgHRR (Sulentic et al., 2004a, 2004b; Fernando et al., manuscript in preparation). However, this study is the first to explore the use of a

human cell line as a model for mechanistic studies on the effects of TCDD on the human hsl,2enhancer of the 3'IgHRR. We found that the IM-9 cell line may be a suitable, but not an ideal model for our 3'IgHRR studies. Our data suggests that IM-9 cells possess a functional AhR pathway, as we identified AhR expression and established TCDD induction of the CYP1A1 gene in IM-9 cells. In a previous study, the expression of AhR was not confirmed in the IM-9 cell line due to conflicting results in which TCDD induced binding of AhR and ARNT protein to a DRE from the CYP1A1 gene, as identified by supershift analysis in an EMSA study, but AhR was not detected by Western blot (Masten and Shiverick, 1996). We believe our confirmation of AhR expression in IM-9 cells can be attributed to increased specificity and affinity of anti-AhR antibody since the time of the previous IM-9 cell study (Masten and Shiverick, 1996). Still, identifying AhR required us to load high concentrations of total IM-9 protein in our Western blot assay, and the expression level of AhR coincided with the considerably lower induction of CYP1A1 when compared to what is seen in the mouse CH12.LX cells (Sulentic et al., 2000). The lowered induction of CYP1A1 supports the theory that human AhR has lower expression and binding affinity for TCDD than mouse AhR (Harper et al., 1988; Ema et al., 1994; Reviewed by Connor and Aylward, 2006) which may influence the level of TCDD-induced CYP1A1 expression and resultant toxic effects. Interestingly, our results contrast with the Nohara et al. study (2006) which suggested that TCDD induced higher levels of CYP1A1 induction in primary human lymphocytes versus AhR high affinity C57BL/6 (C57) mouse cells. However, differences in CYP1A1 induction are related to AhR expression and several polymorphisms of human AhR with varied binding affinity to TCDD have demonstrated cell-type specificity in human tonsils, hepatocytes, placenta

(Lorenzen and Okey, 1991; Lesca et al., 1994; Okey et al 1997). Thus, AhR binding affinity to TCDD may differ between primary lymphocytes and the IM-9 B cell line. Additionally, several laboratories have demonstrated that lowered expression of CYP1A1 could also be dependent on several factors along the AhR pathway other than AhR affinity, including coactivators (Fuji-Kuriyama and Mimura, 2005; Hankinson, 2005; Kim and Stallcup, 2004), chaperone proteins (Carlson and Perdew, 2002; Cox and Miller, 2003), and cross-talk between AhR and other transcription factors (Beishlag and Perdew, 2005; Marlowe and Puga, 2002).

In parallel with our previous findings that TCDD-treatment may inhibit Ig expression and secretion in activated mouse CH12.LX cells, possibly through the AhR pathway (Sulentic et al., 1998, 2000), we hypothesize that TCDD may also inhibit Ig secretion in an activated human cell line. In order to analyze TCDD-induced differences in Ig expression and secretion, an ideal human cell model should express basal and/or inducible Ig. We evaluated basal Ig in IM-9 cells and found a level of basal Ig secretion that was contrary to what was previously reported by the Fahey et al. study (1971), in which IM-9 cells were characterized as high Ig secreting lymphoblast but not plasma cells. In their study they utilized a complex culturing pattern in order to induce high amounts of basal IgG. We followed the culturing pattern illustrated in their 1971 study (Fahey et al., 1971), however their resulting antibody secretion was orders of magnitude higher than our findings, (hundreds of ng per 10^6 cells versus ng per 10^6 cells). This quantitative difference in IgG expression was perplexing, and could have been due to unspecified culturing practices or IM-9 cell line differentiation. The lymphoblast phase of development means that the B cell has encountered antigen but has not differentiated into

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plasma cells which would be demonstrated by high amounts of endoplasmic reticulum within the cell and high levels of Ig secretion. Even though the IM-9 cells in the Fahey study (1971) started off in a lymphoblast stage of development, their IM-9 cell cultures were maintained at considerably high cell concentrations ($2 - 3 \times 10^6$ C/mL) for extended periods of time (10 -12 days). Perhaps, several days of such high cell culture concentrations are necessary to initiate an antibody response in IM-9 cells. However, these same conditions could have led to differentiation of the Fahey IM-9 cells into plasma cells, and concordantly the high levels of Ig that we did not see. Interestingly, in our 96 h Ig secretion studies in IM-9 cells we saw low but increasing numbers of larger than average cells with cellular structures visible at a 1000X magnification, possibly endoplasmic reticulum, after 60 h that would suggest the onset of cellular differentiation. One report has suggested that change in phenotype of B cells during extended periods of culture may be due to interactions with stimulatory fragments of Ig Fc regions that have been secreted into cell media (Hobbs et al., 1985, 1989). Contamination is also a possibility, as one report stated that one of the biggest problems encountered in cell culture is infection with mycoplasmas (Drexler et al., 1999), which has also been associated with differentiation of B cells (Sitia et al., 2005). Alternatively to a possible phenotype change within the Fahey study (1971), our initial culture of IM-9 cells from ATCC could have a different phenotype with respect to the reported phenotype of the IM-9 cells. For example, the IM-9 cells we cultured may have already differentiated into memory B cells, and studies with *in vivo* mouse B cells have suggested that memory B cells may only secrete high levels of IgG if activated by cell specific antigen in addition to TLR activation (Richard et al., 2008; reviewed by Lanzavecchia and Sallusto, 2009). This

may be why they were not activated to secrete Ig by the TLR 9 agonist CpG or the TLR7 agonist R848 which have both reported to be activators of B cell lines (Bauer et al., 2001; Bishop et al., 2001). In light of this premise the IM-9 cells may be activated only if they are treated with the TLR9 agonist CpG or TLR7 agonist R848 in conjunction with, anti-IgG, anti CD40 or CD40L, and/or stimulatory cytokines (i.e. IL-4 or IL-9). Thus, the limited IgG expression in IM-9 cells could hinder determining TCDD-induced effects on Ig expression which makes IM-9 the cell line a less than ideal human B-cell model. However, the IM-9 cells still have a functional AhR pathway which supports the use of them for mechanistic studies of TCDD in the transcriptional regulation of the 3'IgHRR.

The human polymorphic hs1,2 enhancer is activated by TCDD in human IM-9 cells

As stated above, the AhR pathway is thought to end in AhR/ARNT binding to DRE motifs in promoter regions of several genes (Whitlock, 1990). We previously identified DRE motifs within the hs4 and the mouse and human hs1,2 enhancers of the 3'IgHRR which may be sensitive targets of TCDD. More specifically, we found that TCDD activates the mouse hs4, but inhibits the mouse 3'IgHRR. Additionally, although the human and mouse hs1,2 enhancers share a 90% similarity (Mills et al., 1997), TCDD inhibits the activity of a reporter consisting of the mouse hs1,2 enhancer, but activates the human hs1,2 reporter in LPS activated and TCDD-treated IM-9 cells (Sulentic et al., 2000; Fernando et al., manuscript in preparation). This study resolves the question of whether this divergence in transcriptional activation between the mouse and human hs1,2 enhancers in mouse CH12.LX cells was cell model related, by demonstrating that

reporters consisting of the human polymorphic hs1,2 enhancer are also activated by TCDD in IM-9 cells. However the TCDD-induced activation seen in human IM-9 cells did not mirror what was seen in mouse CH12.LX cells. One key difference was that the basal activity of each of the human hs1,2 reporters was magnitudes higher than the activity seen in the CH12.LX cells. This difference could have been cell model related in that human IM-9 cells may possess cellular machinery (i.e. RNA polymerase, basal transcription factors) that is more equipped to activate the human hs1,2 reporters, as mouse cellular machinery may more effectively activate the mouse hs1,2 reporter. This theory is supported by a study from Chen and Birshtein (1997) in which they observed that reporters consisting of the human $C\alpha_1$ 3'IgHRR and $C\alpha_2$ 3'IgHRR demonstrated more basal activity than the mouse hs1,2 enhancer in a human plasma cell line. On the other hand, the mouse hs1,2 enhancer was more basally activated in a mouse plasma cell line than the human $C\alpha_1$ and $C\alpha_2$ 3'IgHRR (Chen and Birshtein, 1997). Whereas the human hs1,2 reporters demonstrated the greatest basal activity in human IM-9 cells, the greatest fold change in TCDD-induced activation of the human hs1,2 reporters was demonstrated in mouse CH12.LX cells. This difference could have also been cell model related in that the AhR has been found to be species specific in several animal models including, mouse, guinea pig, rat and others (reviewed by Connor and Aylward, 2006). In most cases human AhR has demonstrated considerably lower binding affinities and expression levels than other animal models (reviewed by Connor and Aylward, 2006), which may have influenced the extent of TCDD-induced inhibition. Although we saw differences in levels of basal activity and in TCDD-induced fold-activation of the human hs1,2 reporters in IM-9 and CH12.LX cells, we found that the α 1B reporter demonstrated

the greatest TCDD-induced fold-activation in both IM-9 and CH12.LX cells. This finding is relevant because of the multiple autoimmune diseases that have been associated with the expression of the α 1B enhancer of the hs1,2 enhancer, including: IgA nephropathy (IgAN) (Aupetit et al., 2000), Celiac disease, systemic sclerosis, schizophrenia, dermatitis herpetiformis, plaque psoriasis (Frezza et al., 2004,2007, 2009), psoriatic arthritis (Cianci et al., 2008), and rheumatoid arthritis (Tolusso et al., 2009). Several studies suggest a correlation with the α 1B allele of the hs1,2 enhancer and immune deregulation of the IgA1 isotype which relates to our work in that we believe TCDD is a modulator of immune regulation through the 3'IgHRR, and we have shown altered regulation of Ig expression and function by TCDD (Sulentic et al., 1998, 2000). As discussed above we did not identify an ideal stimulant for IM-9 cells so we were unable to compare the effects of TCDD in activated CH12.LX and activated IM-9 cells. However, since the human hs1,2 reporters still demonstrated TCDD-induced activation in IM-9 cells as well as in unstimulated mouse CH12.LX cell, these results are significant because they support the applicability of TCDD mechanistic studies of the 3'IgHRR in mouse cell lines to human application.

BSAP negatively regulates the α 1A+BSAP reporter in IM-9 cells treated with TCDD

As previously stated, the mouse and human hs1,2 enhancers share a 90% homology (Mills et al., 1997). However the mouse and human hs1,2 enhancers have a divergence in transcriptional regulation in which the mouse hs1,2 enhancer is inhibited

by TCDD-induced and LPS co-treated CH12.LX cells, whereas the human hs1,2 enhancer is activated. In the study above we determined that this divergence is not due to differences in cellular models as TCDD activated the human hs1,2 enhancer in both mouse CH12.LX cells and human IM-9 cells. Therefore, we hypothesized that minor sequence differences could be causing the divergence in transcriptional regulation. We utilized the α 1A+BSAP reporter, which is the α 1A reporter (which has no BSAP site) with an added BSAP sequence identical to the high affinity BSAP sequence of the mouse hs1,2 enhancer, in order to explore whether the high affinity BSAP site of the mouse hs1,2 enhancer is the repressive factor causing the divergence in transcriptional regulation of the mouse versus human hs1,2 enhancers. We found that the added BSAP site to the α 1A reporter lowered TCDD-induced activation compared to the wild type α 1A reporter, which suggests that BSAP exerts an inhibitory role on the α 1A+BSAP reporter. These results provide supportive evidence that BSAP may, in part, be contributing to the TCDD-induced inhibition of the mouse hs1,2 enhancer (contains a BSAP site) in LPS-activated CH12.LX B cells as opposed to the TCDD-induced activation of the human hs1,2 enhancer (no BSAP site). This hypothesis is also supported by preliminary studies in our lab in which the α 1A+BSAP enhancer compared to the α 1A reporter demonstrates a significant decrease in total activity in the mouse CH12.LX cells (Ochs et al., unpublished data). BSAP inhibition could have occurred through the recruitment of Groucho family proteins, which are co-repressive proteins reported to localize with BSAP and repress gene transcription (reviewed by Fisher and Caudy, 1998; Parkhurst, 1998). Additionally, BSAP could have competed for recruitment of general transcription factors (TBP and TFIIF), which AhR has also been shown to recruit

(Rowlands et al., 1996). Interestingly, the total activation of the human hs1,2 enhancer was not completely ablated by the addition of the BSAP site in either cell line. This finding may have been due to the collective activation by NF κ B, AP-1, and Oct that we found in our previous site directed mutational studies on the human hs1,2 reporter in LPS and TCDD co-treated CH12.LX cells (Fernando et al., manuscript in preparation). Notably in our earlier studies we also found that the DRE exerts a slightly inhibitory effect (Fernando et al., manuscript in preparation) on the human hs1,2 reporter. Importantly, inhibition of the mouse hs1,2 enhancer may have been due to additional factors besides the high affinity BSAP site we added to the α 1A reporter. For instance, a second low affinity BSAP site is located on the mouse hs1,2 enhancer (Singh and Birshstein, 1993; Mills et al., 1997), and full repression of basal activity of the hs1,2 enhancer has been reported to be seen only when both BSAP sites are occupied (Singh and Birshstein, 1996). In addition, an NF α P site, an ETS-like family motif and positive regulator of the mouse hs1,2 enhancer, is located adjacent to a BSAP site of the mouse hs1,2 enhancer (Neurath et al., 1995). NF α P has demonstrated reciprocal binding with the high affinity BSAP site. More specifically, in mature B cells, binding of the BSAP site of the mouse hs1,2 enhancer blocks the NF α P site and, therefore, inhibits the basal activity of the mouse hs1,2 enhancer (Neurath et al., 1995). On the other hand, in plasma cells, where BSAP is not expressed, binding to NF α P is not blocked which increases the activity of the mouse hs1,2 enhancer resulting in increased mRNA transcripts (Neurath et al., 1995). However, while this may explain a possible mechanism of the BSAP-mediated repression of the mouse hs1,2 enhancer, it does not explain the BSAP-mediated repression we saw of the human α 1A+BSAP reporter, as the human hs1,2 enhancer does

not contain a NF α P site. Although the mechanism behind the inhibition of the mouse hs1,2 enhancer remains unclear, our data strongly supports the theory that BSAP is a primary mediator behind the transcriptional divergence between the mouse and human hs1,2 enhancers.

CONCLUSION

Our results support the use of the IM-9 cell line as a model for mechanistic studies on the effects of TCDD on the human 3'IgHRR, as evidenced by AhR expression and TCDD-mediated induction of the CYP1A1 gene. Our IM-9 cells demonstrated a different phenotype than previously reported in that they expressed only low levels of IgG. Moreover IM-9 cells were not activated by CpG, through TLR9, or R848, through TLR7. However, since increased AhR expression may result in increased B cell sensitivity to TCDD and activation of the AhR pathway, in our future studies we may attempt to activate IM-9 cells with multiple T-independent and dependant agonist, such as a TLR agonist in addition to CD40L, anti-IgG, and/or stimulatory cytokines. Although our IM-9 cells only secrete low levels of IgG, they still have the necessary cellular machinery to facilitate TCDD-induced expression of the human polymorphic hs1,2 enhancer. Moreover we found that the human hs1,2 reporter showed significantly higher basal activation in the human IM-9 cell line than in the mouse CH12.LX cell line. Yet, TCDD induced a greater fold activation of the human hs1,2 reporter in the mouse CH12.LX cell line versus the human IM-9 cell line. Notably, the α 1B reporter demonstrated the greatest TCDD-induced activation in both cell lines. This finding is relevant to human health in that immune deregulation of the α 1B allele of the human hs1,2 enhancer has been associated with several human autoimmune diseases and TCDD-induced modulation of the α 1B allele via the AhR pathway could be one mechanism in

the etiology of the reported diseases. Regardless of the differences in level of basal and TCDD-induced activity, our findings of TCDD-induced activation of the human hs1,2 reporter in both IM-9 and CH12.LX cell lines support the use of the human IM-9 cell line for mechanistic studies of TCDD-induced effects of the human hs1,2 enhancer. One future study that could further validate this conclusion would be to transfect the mouse hs1,2 reporter into human IM-9 cells in order to compare the trend in TCDD-induced effects to our previous observations in the mouse CH12.LX cell line. In addition to demonstrating the similarities and differences in the activity of the human hs1,2 reporter transfected into IM-9 and CH12.LX cell lines, we demonstrated that the BSAP site in the mouse hs1,2 enhancer may be contributing to the inhibitory effect of TCDD on the mouse versus human hs1,2 reporter. This theory is supported by the decrease in TCDD-induced activation of the α 1A allele of the human hs1,2 enhancer with the addition of a BSAP binding site. To study this further we could explore the effects of deleting the high affinity BSAP site from the mouse hs1,2 reporter and transfect this mutated reporter into the mouse CH12.LX cell line. In conclusion, our results that TCDD modulates the activity of the human hs1,2 enhancer in the human IM-9 cell line are highly relevant due to the fact that TCDD is environmentally persistent, and the reports that the AhR pathway is activated by several exogenous and endogenous chemicals and pharmaceuticals in addition to TCDD (Hensler et al., 2009; Flaveny et al., 2009), thus warranting continued study on the impact of TCDD on the human 3'IgHRR in the human IM-9 cell line.

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